Investigating Treatments for Hemorrhagic Stroke Using Rodent Models

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Neuroscience

University of Alberta

Abstract

Intracerebral hemorrhage (ICH), when a blood vessel ruptures within the brain, affects approximately 15% of stroke victims in Canada. There are no proven neuroprotective treatments ICH but two therapies, therapeutic hypothermia (TH) and iron-chelators, have gained preclinical and clinical interest. Therapeutic hypothermia involves cooling the whole-body or brain to typically 32-35°C and has robust neuroprotective effects for neonatal hypoxic-ischemic encephalopathy and cardiac arrest. However, the literature on treating ICH is inconclusive. Cooling lessens inflammation, edema and blood brain barrier damage after ICH; yet this has not consistently led to neuroprotection or functional benefit. To better understand these conflicting reports, this thesis investigated whether TH 1) does not mitigate key mechanisms of injury following ICH and/or 2) causes side effects that negate protective effects. Also, since iron-induced injury is a popular target in preclinical studies and causes greater neuronal death in ICH compared to other types of brain injury that TH protects against, the iron-chelator bipyridine was also evaluated. Our aim was to better understand the effects of TH on specific mechanisms of injury and evaluate a potential therapy that could then be studied in the future as a combination therapy with TH (e.g., an iron-chelator).

The first two sets of experiments used simplistic models of injury to directly study the impact of TH on thrombin- and iron-induced injury. Thrombin is an important clotting factor that prevents on-going bleeding, but is also neurotoxic causing cell death, inflammation and edema. In the first study, thrombin was infused into the rats' striatum followed by TH or maintained normothermia. TH treated rats had a greater number of degenerating neurons two weeks post-infusion but there was no effect on overall tissue loss, edema and functional impairment. This suggested that TH delays but does not prevent ongoing thrombin-induced neuronal damage. In the second study,

$FeCl_2$ was infused into the striatum to evaluate the influence of TH on iron-induced injury. This model of injury causes tissue loss and neurodegeneration, edema, functional impairment and a small amount of intracerebral bleeding. Therapeutic hypothermia significantly reduced bleeding after $FeCl_2$ infusion but had no beneficial effect on any other measure. These results suggest that two key mechanisms of damage after ICH are not treated by TH.

The third study focused on inflammation and hematoma resolution using a rodent model of ICH. It is well known that TH is a potent anti-inflammatory which can be protective, however, we hypothesized that this influence on inflammation would hamper the brain's natural defenses for containing and resolving the hematoma. Interestingly, despite a considerable reduction in inflammatory cells, TH did not lead to a greater spread of iron into the perihematoma zone nor did it impact the rise in non-heme iron, an indirect measurement of hematoma resolution. Most importantly, we found that 40% of treated animals had significantly greater amounts of blood volume a week following stroke (i.e., 3 days following end of rewarming). At time points when treated animals were still being cooled, there was no increase in blood volume in treated groups compared to controls. These data suggest that TH does not impair endogenous mechanisms of hematoma containment and resolution but likely causes rebleeding following either treatment or rewarming in some animals. This substantial increase in blood volume, even in only a few animals, would likely counteract our ability to detect protective effects of TH in group averages. The intermittent nature of the rebleeding could explain the inconsistent neuroprotection seen in the literature and has important implications for clinical use.

In the last set of experiments, bipyridine was investigated using two rodent models of ICH, collagenase and whole-blood, and the simplistic $FeCl_2$ model. The collagenase model causes on-going bleeding by breaking down blood vessel walls. Bipyridine did not impact non-heme iron

levels, edema or functional impairments in this model. In the whole-blood model, a bolus injection of autologous blood, bipyridine was not protective. As well, bipyridine did not impact $FeCl_2$-induced tissue loss, neurodegeneration or behavioural impairment. Bipyridine did cause significant, transient hypothermia. Despite thorough evaluation of bipyridine, we found no beneficial effects, which suggests to us that bipyridine would not be a suitable therapy to combine with TH. In conclusion, TH did not effectively treat thrombin- or iron-induced injury and may cause re-bleeding complications.

Preface

This thesis is an original work that has been given research ethics approval from the University of Alberta Animal Care and Use Committee for Biological Sciences, "Rodent Stroke Studies" No. AUP 960. This project approval was for the duration of all thesis work and beyond.

Chapter 2 of this thesis has been published as Wowk S, Ma Y, and Colbourne F., "Mild Therapeutic Hypothermia Does Not Reduce Thrombin-Induced Brain Injury", in the journal Therapeutic Hypothermia and Temperature Management (2014), volume 4, pages 180-187. Wowk S was responsible for developing the experimental rationale and design of this study and for data collection and analysis and manuscript writing and editing. Ma Y assisted with data collection and analysis. Colbourne F was the supervising author and lead and responsible for experimental design, manuscript editing and project supervision. Doi: 10.1089/ther.2014.0014

Chapter 3 of this thesis has been published as Wowk S, Ma Y, and Colbourne F., "Therapeutic Hypothermia Does Not Mitigate Iron-Induced Injury in Rat", in the journal Therapeutic Hypothermia and Temperature Management (2016), volume 6, pages23-29. Wowk S was responsible in forming the rational and the design of the experiments, as well as, for data collection and analysis and manuscript writing and editing. Ma Y assisted with data collection. Colbourne F was the supervising author and responsible for experimental design, manuscript editing and project supervision. Doi: 10.1089/ther.2015.0020.

Chapter 4 of this thesis has been published as Wowk S, Fagan K, Ma Y, Nichol H, and Colbourne F., "Examining Potential Side Effects of Therapeutic Hypothermia in Experimental Intracerebral Hemorrhage", in the Journal of Cerebral Blood Flow & Metabolism (2017), volume 37, pages 2975-2986. Wowk S was responsible in forming the rationale and experimental design

and for data collection and analysis and manuscript writing and editing. Fagan K and Ma Y were involved in data collection and Fagan K was also involved in data analysis and manuscript editing. Nichol H aided in experimental design and data collection and provided supervision and consultation for the synchrotron experiment as well as manuscript editing. The imaging portion of Chapter 4 was part of an international collaboration CIHR team grant under Nichol H. Colbourne F was the supervisory author and was responsible for experimental rationale, design, manuscript editing and project supervision. Doi: 10.1177/0271678X16681312

Chapter 5 of this thesis was co-first authored and published as Caliaperumal J, Wowk S, Jones S, Ma Y, Colbourne F., "Bipyridine, an Iron Chelator, Does Not Lessen Intracerebral Iron-Induced Damage or Improve Outcome After Intracerebral Hemorrhagic Stroke in Rats", in the journal Translational Stroke Research (2013), volume 4, pages 719-728. Wowk S was responsible for experimental rationale and design development, project management and scheduling, data collection and analysis, and manuscript writing and editing. Specifically, Wowk S was responsible for the project management and scheduling duties, telemetry probe surgeries, data collection and analysis of assay work, writing of the introduction, methods, and discussion for the manuscript and final editing of all sections. Caliaperumal J was responsible for experimental rational and design development, data collection and analysis, and manuscript writing and editing. Specifically, Caliaperumal J was responsible for behavioural data collection and analysis, data collection and analysis of assay work, and writing of the results and figure sections of the manuscript. Wowk S and Caliaperumal J jointly and equally shared responsibilities related to the development of the experimental rationale. Jones S was involved in experimental rationale development and was involved in data collection, analysis and manuscript editing. Ma Y was involved in data collection. Colbourne F was the supervising author and responsible for

experimental rationale and design development, manuscript editing and project supervision. Doi: 10.1007/s12975-013-0272-3

Dedication

This thesis is dedicated to Andrew, Dwayne, Suzanne, Kaylee, Scott and Ana for their unwavering support.

Acknowledgements

I would like to acknowledge the overwhelming support I have received from my supervisor Fred Colbourne, who has encouraged me to think critically and plan carefully, skills I will carry with me. I would like to thank my committee members for their expertise and for helping me to always consider clinical relevance in my research, an essential perspective in preclinical research.

As well I would like to acknowledge my lab mates, in particular Ana, as well as my husband Andy and my family and friends who have been a never-ending source of encouragement and much needed laughter. Thank you everyone for your unwavering support.

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List of Abbreviations

BBB	Blood Brain Barrier
BIP	Bipyridine treated group
BP	Blood Pressure
BWC	Brain Water Content
CBF	Cerebral Blood Flow
Cereb	Cerebellum
Crt	Cortical
CONTRA	Contralateral
CTT	Corner Turn Test
Expt	Experiment
FJ+	Fluoro-Jade positive
HIE	Hypoxic-ischemic Encephalopathy
HO-1	Heme-oxygenase 1
HYPO	Hypothermic treated group
ICH	Intracerebral Hemorrhage
ICP	Intracranial Pressure
IPSI	Ipsilateral
NDS	Neurological Deficit Scale
NORMO	Normothermia control group
PPAR-γ	Peroxisome proliferator-activated receptor gamma
rFVIIa	Recombinant Factor VIIa
SAH	Subarachnoid Hemorrhage

SAL	Saline control group
SCI	Spinal Cord Injury
SD	Standard Deviation
TBI	Traumatic Brain Injury
TH	Therapeutic Hypothermia
XFI	X-ray Fluorescence Imaging

Chapter One

General Introduction

1.1 Introduction

Over the past few decades, incredible progress has been made for cardiovascular diseases such as heart disease and stroke. Research into the prevention and treatment of cardiovascular diseases has led to a considerable decrease in the number of affected Canadians.[1] But despite these advances there is more work to be done, as according to the Heart and Stroke Foundation of Canada, heart disease and stroke are still major contributors to death, hospitalization, and disability in Canada. In fact, currently one Canadian dies every 7 minutes due to heart attack or stroke with stroke being the third leading cause of death in Canada.[1] Therefore, especially in cases such as hemorrhagic stroke where there are still no therapies to treat victims and protect brain tissue, more research is needed to improve outcomes for those afflicted with cardiovascular diseases.

1.2 Stroke

Stroke, an interruption of blood flow to an area of the brain, can be categorized into two main subtypes, ischemic and hemorrhagic stroke. Ischemic stroke is due a blockage of blood flow resulting from an embolism or thrombosis. Hemorrhagic stroke is due to a ruptured blood vessel which can occur within brain tissue (i.e., intracerebral hemorrhage (ICH)), in the subarachnoid space (i.e., subarachnoid hemorrhage (SAH)), or in the ventricles. While both ischemic and hemorrhagic strokes are devastating and can lead to mortality and permanent impairments, hemorrhagic stroke is associated with a greater 30-day mortality and lasting impairments.[2,3] Of the types of hemorrhagic stroke, ICH is the most prevalent and is the focus of this thesis.

1.3 Intracerebral Hemorrhage (ICH)

Intracerebral hemorrhage is the second most common stroke accounting for 15-30% of all strokes[2] and is associated with a 30-day mortality rate between 30-44%.[3,4] This mortality rate is predicated by hemorrhage volume, and in fact those with a large hemorrhage (i.e., volume ≥60 cm^3) have a 91% risk of mortality.[4] There are also high degrees of persistent impairments in ICH survivors[2,4] which increase the dependency of these survivors leading to high direct (e.g., rehabilitation) and indirect effects and costs (e.g., caregiver, workplace absenteeism).[5] Unfortunately, there are no approved therapies for treating ICH with only medical management and rehabilitation to aid patients.[6]

A number of risk factors are associated with intracerebral hemorrhage. These include hypertension, family history of ICH, a personal history of ischemic stroke, gender, age, race, the genotypes APOE ε2 and ε4, and warfarin use.[7,8] Certain risk factors also influence the likelihood of where the hemorrhage will occur. For instance, the APOE ε2 and ε4 genotypes and the development of cerebral amyloid angiopathy is associated with lobar hemorrhages (i.e., hemorrhages occurring in white matter of the cerebrum or at the junction between white and grey matter).[8,9] Hypertension (i.e., abnormally high levels of blood pressure (BP)), which is common in all types, is more associated with deep structure hemorrhages such as the basal ganglia including the thalamus and striatum).[8] Although anticoagulant therapies such as warfarin are associated with increased risk of ICH they are also a standard therapy for those at risk or who have had an ischemic stroke.[10] Warfarin not only increases the risk of ICH but also the risk of hematoma expansion (i.e., ongoing bleeding), worsened outcomes and death.[10-12] Hypertension is the primary risk factor for ICH and much work has been and is currently being done to

understand how managing BP influences patient outcomes (please see section 1.3.1 for more information).

1.3.1 Current Medical Management

Unfortunately, there are no well established therapies to treat ongoing brain damage after an ICH and current practices rely on medical management to treat patients. This care during hospital stay includes monitoring and management of certain factors including fever, clinical seizures, BP, intracranial pressure (ICP), cerebral perfusion pressure, hemodynamics, and complications such as dysphagia, cardiac events and infections.[6] Typically, the majority of the bleeding of an ICH ceases within 3 hours but in 26-35% of patients there is ongoing hematoma expansion that increases by at least a third.[13,14] Computed tomography angiography is a useful imaging tool that can pick up spot signs (indication of an area with on-going bleeding) and more spot signs are predictive of a greater risk of hematoma enlargement.[6,15] Hematoma enlargement is associated with a greater mortality and a worsened outcome;[6] however, therapies for enhancing coagulation have proved to be ineffective. Phase III clinical trials of recombinant factor VIIa (rFVIIa) were successful at limiting hematoma volume but this did not lead to improved outcomes.[16] Fever is also associated with a poorer outcome and hematoma growth,[17] but there is no clear clinical and preclinical evidence on the influence of temperature management on outcome.[6,18,19] As well, high systolic BP is implicated in causing hematoma expansion, which has in part prompted the recommendation for early BP management even prior to patient transfer out of the emergency department.[6]

As previously mentioned, hypertension is a common risk factor for ICH but there has been concern as to whether aggressive BP decreases may lead to ischemia in the brain. Generally, lower levels of BP are associated with lower mortality and better outcome,[20] but clinical trials

lowering high BP, did not improve mortality or functional outcomes.[21,22] Further exploratory analyses of the ATACH II trial found that while intensive BP reduction led to a reduction in hematoma expansion in deep ICH this did not lead to an improved outcome.[23] This may be due to the intensity of blood-pressure lowering causing ischemic injury in regions of the brain.[24] The data are inconclusive on this matter with some studies finding development of small ischemic lesions[25,26] while others fail to find a significant ischemic insult occurring after an ICH.[6] Several trials (e.g., INTERACT II and ATACH) have now found that early, intensive lowering of systolic BP to less than 140 mmHg is safe at least in small to medium size hemorrhages in patients with moderate hypertension[6,27] with hematoma volume, abnormal changes in white matter and microbleeds being bigger risk factors for ischemic lesions following ICH.[25] While these trials show safety, more trials are needed to determine whether aggressive lowering of BP improves outcome and a prospective phase II clinical trial (ADAPT II) is currently underway.[28] As well, for those exhibiting very high BP (>220 mmHg) and/or large hemorrhages, data are still needed to determine safety and efficacy of intensive BP lowering.

Anticoagulant and antiplatelet therapies are also an important risk factor for ICH that may impact outcome. There is inconsistent evidence that anti-platelet therapies affect hematoma expansion.[6] Like lowering of BP, it is recommended that counteracting these therapies occur early after ictus.[6] This is done by reversal therapies such as infusions of vitamin K plus fresh frozen plasma infusions or coagulation factors such as pro-thrombin complex concentrates or rFVIIa.[6,16,29]

Common serious medical complications that arise after an ICH include dysphagia, aspiration and pneumonia.[6] Dysphagia is the difficulty in swallowing and aspiration occurs when saliva or foreign substances such as water or food are breathed into the respiratory tract. Both of these complications are risk factors for pneumonia, the most common complication.[6,30] Monitoring

dysphagia and aspiration after stroke through early screening substantially reduces the risk for pneumonia.[6,30]

Although it is difficult and invasive to directly monitor ICP, in certain situations it may be necessary such as when a patient has considerable intraventricular hemorrhage, a poor outcome score on the Glasgow coma scale, and/or is starting to herniate.[6] An increase in ICP is more common in young patients or supratentorial hemorrhages and the resulting hydrocephalus is associated with a poor outcome.[6] To measure ICP directly a monitor can be implanted either directly into brain tissue or in a ventricle.[6] While a catheter into the ventricle can also allow for drainage of cerebrospinal fluid to decrease pressure it can also cause intraventricular hemorrhaging.[6] There are several ways to mitigate increasing ICP including elevating the head to 30°, mild sedation, mannitol or hypertonic saline, cerebrospinal fluid drainage, hematoma evacuation, or decompressive craniectomy but no studies have shown efficacy of ICP management.[6] Both hematoma evacuation and decompressive craniectomy are beneficial in life-saving situations but there is no clear evidence of benefit especially in the case of supratentorial hemorrhage.

1.3.2 Pathophysiology

There are several mechanisms that contribute to cell atrophy and death after an ICH. Primary injury, damage due to the mechanical force of blood tearing through brain tissue, occurs quickly in the minutes to first few hours after the initial bleed and contributes substantially to the damage after an ICH. As seen in the clinical literature, ~30% of patients will have hematoma expansion over the ~24 hours after hemorrhaging begins, although the majority occurs within the first few

hours, which increases the risks of mortality and worsened impairments.[13-15] The mass effect of bleeding and rising edema levels can lead to an increase in ICP, which can further contribute to damage and if high enough, such as in large hemorrhages, can cause herniation and mortality[20] and a decrease in cerebral perfusion pressure. A decrease in cerebral perfusion pressure may lead to focal ischemic lesions in the surrounding tissue although the data on this is limited.[6,31] Management practices for mitigating primary damage are currently used (e.g., BP control, anticoagulant reversal therapies); however, the typically quick nature of this damage makes targeting primary injury difficult.

Secondary injury occurs in the hours to weeks following the initial bleed. This protracted time course of injury and cell death provides potential targets for neuroprotective therapies. There are several mechanisms of secondary injury that occur after ICH including inflammation, edema, clotting factors, excitotoxicity, seizures, blood and erythrocyte components, oxidative stress and blood brain barrier (BBB) disruption.[32-34] These mechanisms contribute to protracted cell death that can occur over the hours to weeks after the initial bleed. The majority of our understanding of injury after ICH is from animal work and a few clinical studies, and there is still much that needs to be understood on the nature of injury after ICH. For more information on the animal models used to understand ICH pathophysiology and potential treatments please refer to section 1.3.3. This thesis has focused on inflammation, clotting factors, and blood constituents as they contribute to hemorrhagic stroke damage in a different manner than ischemic stroke which may impact the effectiveness of TH for ICH.

While thrombin plays a key role in the clotting process and is thus important for mitigating the bleed, it can also be neurotoxic. Thrombin is an enzyme that is a necessary component of the coagulation cascade that is activated soon after bleeding starts. In response to blood vessel

damage, a series of processes in the coagulation pathway occurs and leads to the cleavage of prothrombin to produce thrombin.[32] Thrombin goes on to convert fibrinogen into the insoluble fibrin protein which forms a clot with platelets.[32] Thrombin is essential for curtailing the primary damage of an ICH; however, thrombin itself is neurotoxic and can lead to acute cell atrophy, neuronal death and functional impairments.[33,35] This toxicity is due to thrombin-induced inflammation, metalloproteinase production and BBB disruption, and edema.[32,33,36-38] Antithrombotic agents (e.g., argatroban) and anti-inflammatory drugs (e.g., minocycline) have shown benefit against thrombin-induced injury.[33,37] It should be noted however, that along with clot formation another potential benefit of thrombin is stimulating neurogenesis and angiogenesis,[39,40] both of which may play an important role in recovery after ICH although this is not clear.[41,42]

Inflammation is a necessary response when damage or infection occurs in the body, but like thrombin, inflammation can also play a harmful role after ICH. The inflammatory response contains and clears the damage that occurs but also inevitably injures normal healthy cells due to the broad spectrum response of inflammation. There are several methods to evaluate different aspects of the inflammatory response in preclinical studies such as characterizing cells through histological staining (e.g., Perls' Prussian blue stain for microglia/macrophages, Leder's stain for neutrophils) and immunohistochemistry (e.g., labelling Iba-1 for microglia), as well as, using western blotting and rt-PCR for measuring levels and regulation of inflammatory markers (e.g., cytokines), respectively. This thesis focused on using the Perls' Prussian blue stain to identify and quantify microglia and macrophage cells as it has been previously used and was sufficient for confirming the decrease in inflammation in cooling animals.[18,43,44]

Neutrophils are small white blood cells capable of crossing the BBB and appear within the first hours after an ICH.[33,34,45,46] Other cell types that are part of the early inflammatory response include macrophages that enter through the leaky blood vessels and microglia already in the area.[33,34] Microglia are the brain's resident inflammatory cells that are highly similar to macrophages. Microglia are capable of phagocytosis and releasing pro-inflammatory cytokines and chemokines which aid in proliferating the brain's inflammatory response.[33,34] Macrophages are also capable of crossing the BBB and once activated become undifferentiable from microglia. These cells begin crossing the BBB within the first few hours after the bleed and also release pro-inflammatory molecules.[34] The inflammatory response continues to grow and peaks at about 3 days post-ICH, and inflammatory cells remain elevated in the peri-hematoma for weeks following a stroke.[34,47,48] As part of the inflammatory response, these cells release matrix metalloproteinases which contribute to BBB damage and thus vasogenic edema.[33,37,47] As well, inflammatory cells generate reactive oxygen species causing oxidative stress which leads to damage to the lipid membranes of neurons and other macromolecules and thus contributes to cytotoxic edema, cell atrophy, and neuronal death.[33]

Despite the potentially harmful effects of inflammation, this response is also important for containing and resolving the hematoma. While several studies have found beneficial effects with anti-inflammatory therapeutics, such as minocycline,[49] others have found that peroxisome proliferator-activated receptor game (PPAR-γ) agonists (e.g., rosiglitazone) can be beneficial by quickening hematoma resolution after ICH.[50,51] PPAR-γ is important for regulating the phagocytosis response in inflammatory cells which allows for removal of erythrocytes from the extracellular space before they can fully breakdown and release harmful by-products.[52]

Inflammation is an innate response to mitigate damage, however, its proliferative and general nature can become unmanageable and contribute to neuronal cell death.

Another major component of secondary injury after ICH is the neurotoxicity associated with erythrocytes. While intact erythrocytes themselves may not be wholly toxic, these cells begin to degrade in the first few days after an ICH[32,53,54] and the hemoglobin within these cells is toxic. Hemoglobin consists of globin proteins and iron-containing heme. Hemoglobin is still contained within erythrocytes 24 hours after ICH but begins to be released by two days[54] and peaks in edema following ICH correlate with release of hemoglobin.[54] Once hemoglobin breaks down, heme is further broken down by heme oxygenase, an enzyme produced by inflammatory and endothelial cells.[53] Heme-oxygenase 1 (HO-1) levels are increased in response to heme-hemopexin complexes and heme.[54,55] After ICH, HO-1 levels increase over the first few days, peaking at 3 days but return to normal levels by a month after stroke in rodents.[56] From heme, carbon monoxide, bilirubin, and ferrous iron are released and it is this free form of iron that is potentially most toxic as a pro-oxidant.[33,57,58] Non-heme iron levels, an indirect measure for heme degradation, increase over the first week after ICH and remain elevated in the month following stroke. [59] Iron and hemoglobin toxicity is associated with edema, seizures, inflammation, BBB disruption, and oxidative stress after ICH[57,60-62] and is associated with acute and long term cell death.[62-64] In fact, iron chelators have been a substantial focus of potential therapeutics for ICH with several studies investigating deferoxamine.[33] A more detailed discussion on iron chelators as a treatment for ICH can be found near the end of this chapter (please refer to section 1.5). To study hematoma resolution in this thesis we used two commonly used indirect measures, non-heme iron levels and hematoma volume (i.e., hemoglobin content).

To further evaluate iron, we used x-ray fluorescence imaging to spatially map and quantify iron following ICH which has been previously done in our lab.[59]

The iron released from heme is free to participate in Fenton's reaction where unbound ferrous iron and hydrogen peroxide is converted to ferric iron, a hydroxide ion, and a hydroxyl radical.[65] Hydroxyl radicals are one of the most potent pro-oxidants and substantially contribute to oxidative stress.[65] The brain and body have endogenous mechanisms for mitigating hemoglobin and iron toxicity. Haptoglobin is found in blood plasma and binds free hemoglobin to allow for a safe degradation of hemoglobin without release of free iron.[54] In response to ICH, haptoglobin production by oligodendrocytes increases over the first 3 days and may help mitigate hemoglobin-induced toxicity as mice genetically engineered to over produce haptoglobin have reduced functional deficits compared to wild-type mice.[66] The brain (and body) also has endogenous mechanisms to cope with free iron. Transferrin and its receptor are important for short term storage and transportation of iron. Under normal conditions transferrin and transferrin receptors are regulated in response to cellular needs of iron as transferrin is the main iron transporting protein.[54,67] In the brain, transferrin is produced by oligodendrocytes and is important for binding extracellular ferric ions[54] and transports iron into cells through the transferrin receptor.[54] In the days following ICH, both transferrin and its receptor are upregulated[56] and this likely aids in the transportation of iron out of the extracellular spaces in the brain.[54]

Following ICH, the vast majority of the iron remains in the injured hemisphere as has been seen in both animal models of ICH[56,59,68] and in the clinic.[68] Thus, to avoid chronic iron toxicity there is long term storage of iron via ferritin proteins which amalgamate into hemosiderin deposits. Ferritin binds to ferrous ions and is composed of both a light- and heavy-chain and is an essential

protein found in microglia, oligodendrocytes and epithelial cells.[54,56] The heavy chain acts as a ferrioxidase to convert ferrous ions into the ferric form and the light chain forms the core where ferric molecules can be stored.[54] Ferritin expression is regulated by iron-regulatory proteins, when iron levels are high the binding activity of these proteins to ferritin mRNA is low leading to increased ferritin expression.[54,69] In iron-regulating protein-2 knockout mice, there is greater increase in levels of ferritin following ICH compared to wild-type mice at 3 days-post stroke.[69] The increase in ferritin levels in the knockout mice corresponded with lower levels of protein oxidation following ICH[69] suggesting that increased ferritin expression is protective against oxidative stress. Both increases in iron and heme levels in the brain can lead to increased expression of ferritin.[54] Therefore, it is not surprising that both the H- and L-chains of ferritin are upregulated after an ICH by 3 days and remain at high levels by a week post ICH.[56] Ferritin levels drop off but remain elevated by 28 days after ICH in rats.[56] Thus, since iron has a great capacity to cause damage there are endogenous mechanisms to mitigate iron damage.

As previously mentioned, iron can propagate oxidative stress through the creation of reactive oxygen species but there are also endogenous antioxidants, such as superoxide dismutase, catalase, and glutathione, to moderate oxidative damage. By 24 hours following ICH in rats, there is a 50% increase in reactive oxygen species in the injured hemisphere compared to the contralateral striatum.[70] In one preclinical study, hydroxyl radical levels were increased above sham levels by 6 hours post-ICH and remained above sham levels for at least two weeks.[71] In rodents, lipid peroxidation and DNA damage increases by 6 hours post-ICH, peaks at 3 days and decreases but remains elevated for at least two weeks.[71,72] Nakamura and others[61] found a similar trend for DNA and protein oxidation with increases seen over the first few days and above normal levels persisting for at least a week-post ICH in rats. Expression of Cu/Zn superoxide

dismutase increases starting at 3 days post-ICH, peaks at day 7 and begins to decrease by 14 days[72] to mitigate this oxidative stress. In preclinical studies, treatment with antioxidants can diminish increases in hydroxyl radicals and oxidative stress[71,73] although the antioxidant NXY-059 unfortunately did not pass clinical trials.[74] While there are endogenous mechanisms for managing hemoglobin, iron and oxidative stress, it is likely that these systems are overwhelmed by the volume of blood in a hemorrhage. As well, acidity and oxidative stress, which can stem from other sources such as inflammation and excitotoxicity, can cause release of iron from ferritin and interfere with antioxidant activity.[54] Therefore, much of the preclinical research for hemorrhagic stroke has focused on the hematoma, iron toxicity and oxidative stress.

1.3.3 Animal Models of ICH

There are several animal models used to investigate therapies for and build our understanding of ICH. The two most widely used models are whole-blood and collagenase. In animal studies strokes are typically induced in the striatum as this is a common location in patients.[75,76] The whole blood model involves an injection of autologous blood, typically obtained from the tail artery, into the brain.[76,77] In the collagenase model there is an injection of bacterial collagenase, which degrades blood vessel walls, into the brain parenchyma leading to a bleed.[76,78] While neither model perfectly exemplifies what happens in a human ICH, they both have their uses. The whole-blood model does not involve the injection of a foreign substance and there is no protracted bleeding or seizure activity which also do not occur in many ICH patients.[6] The collagenase model causes a bleed in the brain and is associated with more long term cell death, greater long term impairments, more inflammation, more BBB disruption and a larger increase in ICP compared to the whole-blood model[79,80] as well as seizure activity.[81] Another model used in

experimental ICH studies involves feeding spontaneously hypertensive rats a high salt diet.[82] This model can lead to spontaneous hemorrhage but there is high variability in lesion size and location making it difficult to use in controlled studies.[83]

Other models used to study ICH take a reductionist approach to simplify the study of different components involved in ICH. For instance, several studies have used hemoglobin or hemoglobin components to better understand the role of each mechanism in ICH injury and how treatments influence these mechanisms.[60,66] Intraparenchymal infusions of thrombin are used to help to better understand thrombin-induced injury.[35,36,39,40] Thrombin-infusion causes acute neurodegeneration and behavioural impairments[35] and has been shown to cause inflammation, edema, BBB disruption and seizures.[36,38,84] Infusion of $FeCl_2$ is a reductionist model to understand iron-induced injury in the brain.[57,62,63,81,85] This simplistic model of iron-induced injury has been shown to cause oxidative stress, seizures, edema, inflammation and cell death[57,62,81,85] which leads to on-going neurodegeneration and cell atrophy and lasting behavioural impairments weeks post-infusion.[63] Both the thrombin and iron reductionist models have been previously used in the literature and have been shown to cause cell death and behavioural impairments similar to the whole-blood and collagenase models.

In this thesis we used the whole blood, collagenase, thrombin-infusion and $FeCl_2$ infusion models. Most of the ICH work done used the collagenase model as it causes a bleed within the brain and thus would involve greater thrombin activity due to the significant effect on blood vessel walls as well as greater deficits and cell death. We used the reductionist models of thrombin and $FeCl_2$ to isolate the effects of these types of injury specifically when using therapeutic hypothermia and bipyridine. As with much of the ICH animal literature we produced damage in the striatum as the basal ganglia is an area where ICH commonly occurs in humans.[75]

1.4 Therapeutic Hypothermia (TH)

Therapeutic hypothermia involves cooling the body below normal temperatures to protect the central nervous system from damage. There has been much interest in using TH and temperature management to treat and mitigate damage after several nervous system injuries. For instance, previous stroke studies of some neuroprotective pharmaceuticals, such as MK-801, were found to influence body temperature and when temperature was controlled and normothermia maintained, those drugs lost their neuroprotective effects.[86,87] Currently, TH is used to treat neonatal hypoxic-ischemic encephalopathy (HIE) and cardiac arrest and is being studied preclinically and clinically in other types of brain injury. Typically, TH is applied to the whole body (i.e., systemically), but in neonates it is possible to use TH localised to the brain due to the thinness of the skull at birth.[88] There are presently no clinically available (approved) methods of inducing localised TH to the brain in adults, but there are methods are various stage of development. There is some debate as to what the ideal target temperature should be although the range in most of the TH literature has been between 32-36°C. In this thesis, we have chosen a target temperature of 33°C, considered here as a mild level of TH, as this has provided considerable success in our lab for global and focal ischemia. As well, this is within the range of the target temperature currently used in the clinic for cardiac arrest[89] and neonatal HIE.[88] Additionally, we used systemic TH in this thesis as it is what is currently used in adults in the clinic and they are the population most likely to experience an ICH.

1.4.1 History, Clinical Use and Complications

The use of cooling as a therapy is not a new concept.[90,91] There is documented use of TH for successfully treating ICH in 1848[92] and there was interest in using TH to treat stroke in the early 20th century.[90] In 1958, Howell and colleagues[93] published a series of case studies in which TH was applied to suspected ICH patients. Although the authors noted limited success in reducing mortality, they were hopeful that TH could still be used to lower high levels of ICP. Nevertheless, due to complications such as infection and rewarming issues, interest in cooling's therapeutic value began to wane by the 1970's.[90,91] Fortunately, during the 1980's and '90s interest began to pick up especially for treating traumatic brain injury (TBI), cardiac arrest (and the resulting global ischemia of the brain), ischemic stroke and neo-natal HIE.[91,94-97] It was identified in animal studies that when given for a sufficiently long duration, TH could provide substantial neuroprotection even with a delay after global[98,99] and focal ischemia.[100-102] With this renewed interest, there have been a number of preclinical and clinical studies in the past 30+ years evaluating temperature management, including TH, to treat spinal cord injury (SCI), TBI, ischemic stroke, and hemorrhagic stroke.[91]

Currently, TH is clinically used to treat cardiac arrest and neonatal HIE. According to the 2016 Canadian Guidelines for the use of targeted temperature after cardiac arrest for post-resuscitative care, the typical target temperature range for TH is 32-34°C with rewarming at a rate of 0.25-0.5°C/hour.[89] While there are no recommendations on treatment time windows it was highlighted that the effectiveness of TH diminishes the longer it takes to reach the target therapeutic temperature. Therapeutic hypothermia is recommended for both inpatient and outpatient cardiac arrest and can be initiated by trained paramedics in a pre-hospital setting to start treatment as early as possible.[89] According to the Canadian Pediatric Association, TH is the

only approved neuroprotectant for neonatal HIE.[88] The target temperature for treating HIE in infants ranges between 32.5-35°C with a temperature of 34±0.5°C considered ideal. Cooling should be initiated within 6 hours from birth and is recommended to last between 48-72 hours, although the optimal duration has not been adequately evaluated. The recommended rewarming rate is 0.5°C every 1-4 hours. As previously mentioned, in neonates TH can also be applied selectively to the brain via a cooling caps[88] but the majority of clinics use systemic cooling methods due to low costs and feasibility and this is done via cooling blankets/packs or passive cooling.[88] Although systemic TH is most widely used, there is no clear neuroprotective advantages associated with one method over the other although there is some evidence.[88,103]

There are different methods for inducing systemic TH in adults although it is not clear whether one method is superior to another. These methods have been used in clinical studies for a variety of brain injuries such as ischemic and hemorrhagic stroke, cardiac arrest, TBI, and SCI.[94,96,104-106] One popular method involves cooling the body externally with servo-controlled cooling blankets which are placed over the skin and can be supplemented with alcohol and ice baths.[94,96,107] Endovascular cooling involves implanting a device, such as a tube or balloons, that surround blood vessels (e.g., femoral vein or inferior vena cava) and is flushed with cold fluids.[105,106,108] This method cools the blood which is circulated throughout the body. The RhinoChill Device™ causes intranasal cooling that induces rapid cooling by spraying a perfluorocarbon–oxygen mixture into the nasal cavity which causes evaporative cooling. This method is typically used to induce TH while systemic methods then maintain TH.[107,109] Since it is important to reach the target temperature in cardiac arrest as quickly as possible, the RINSE trial evaluated whether injecting chilled saline into the peritoneal cavity while on the way to the hospital would improve outcomes compared to initiating TH at the hospital.[110] While they were

unable to find a benefit of prehospital induction of TH in that trial, others argue that it may be useful in hospitals without optimized standardized post-cardiac arrest care.[111]

There are a number of adverse effects and complications associated with TH including infections, shivering, cardiac arrhythmias, coagulopathy and rewarming related issues. One of the most common associated adverse outcomes is infections such as pneumonia.[112-116] This stems from the potent anti-inflammatory effects TH has on the body which increase the susceptibility of the patient to contracting an infection or dealing with a pre-existing infection. This is further complicated by the fact that stroke most often occurs in the aged population who may already have compromised immune systems and stroke can further diminish the immune response.[117,118] Another common complication is the innate shivering response of the body to cooling.[91,112,113,116] Shivering increases metabolic demand and causes vasoconstriction thereby impacting the ability to reach target core body temperature.[91,112,116] While shivering can be mitigated through drugs such as meperidine and buspirone,[112,113,116] this may cause other harmful effects such as increased risk of pneumonia due to swallowing impairment and aspiration.[113] As well, the pharmacokinetics of drugs is changed when the body is cooled making it difficult to predict and respond to the drug's actions during cooling.[91] Shivering protocols that involve drugs, skin warming and/or early monitoring can be useful in mitigating this complication.[112,116] Another adverse effect is cardiac arrhythmias such as bradycardia, tachycardia, and arterial fibrillation which may occur during TH or while the patient is undergoing rewarming.[91,114-116] Coagulopathy can occur and increase the risk of bleeding;[114,116,119] however, this is depth dependent with drops below 35°C but above 33°C having an impact on platelet function but temperatures below 33°C further interfering with other aspects of the coagulation cascade such as enzyme clotting action.[119] While coagulopathy is a potential concern, clinical trials have found combining TH

with thrombolytic drugs to be safe in ischemic stroke.[112] Generally, TH should not be used in actively bleeding patients or in patients with severe acidosis.[119]

Finally, there are complications associated with rewarming. Joshi and others[120] found that during rewarming, cerebral blood flow (CBF) autoregulation was impaired in patients undergoing cardiopulmonary bypass surgery and the authors commented that impairment in CBF autoregulation may contribute to risk of stroke. While they did not compare rates of rewarming in this study, it does show a potential complication with rewarming. In one early trial of severe ischemic stroke, 30% of subjects died during TH or rewarming due to rebound increases in ICP with shorter rewarming being associated with a greater increase in ICP. [115] Complication with rewarming can be mitigated by active, slow rewarming as opposed to passive or rapid rewarming[121-124] or by the duration of TH treatment.[125] A preclinical study of TBI found that slow rewarming led to normal vascular responsiveness following treatment while rapid rewarming was associated with impaired vascular responsiveness.[122] Another TBI study found that rats who underwent slower rewarming had significantly less axonal damage than rats who underwent fast rewarming.[123] When rewarming occurs too quickly several adverse events can occur including oxygen consumption and electrolyte imbalance and resulting cardiac and metabolic issues, disrupted glucose homeostasis, impaired CBF, and increased inflammation and oxidative stress.[121,124] Some of these effects, such as the increased inflammation and electrolyte imbalance, may lead to a rebound in edema. In fact, a study from our lab found that rapid rewarming from TH localized to the brain led to an increase in edema after large hemorrhages, with levels even above animals who had been kept normothermic.[126] As well, Jiang and colleagues[125] found that following TBI in patients, ~5 days of TH lessened rebound ICP after rewarming compared to ~2 days of treatment, likely due to lower levels of edema. Therefore,

while rewarming is a known issue, both the duration of TH and rate of rewarming can mitigate the negative impacts of rewarming. Unfortunately, optimal rates of rewarming and duration of TH have not been well studied in stroke patients or preclinical ICH research.

1.4.2 Clinical Trials

While TH has only been approved to treat neuronal injury in cardiac arrest and neonatal HIE, several studies have been conducted to determine the safety and efficacy of using TH in other types of injuries. Despite the side effects mentioned in section 1.4.1, TH does appear to be safe for a variety of brain injuries. For example, Abou-Chebl and others[107] found cooling via the Rhino-Chill Device™ to be safe for patients who had a TBI, ischemic stroke, or ICH. The following section will describe the current clinical literature for using TH to treat SCI, TBI, ischemic stroke, and of most interest for this thesis, intracerebral hemorrhage.

While there is currently not enough evidence to warrant recommendation in care management guidelines for SCI, early clinical studies have found TH to be safe for patients who have a SCI.[91,106,127] Madhavan and others[127] found that intravascular cooling for 24 hours immediately post-surgery was safe, with patients experiencing better than expected outcomes. A phase I clinical trial found that SCI patients who received intravascular cooling for 48 hours had a similar complication rate to case-matched controls and this preliminary evidence suggested that TH provides benefit beyond spontaneous recovery.[106] With this early clinical success and positive results in preclinical studies,[128] a randomized controlled trial is being conducted as part of the Miami Project to further investigate intravascular cooling for SCI (Identifier: NCT02991690).

There have been several clinical studies evaluating TH for TBI with guideline recommendations for using TH for at least 48 hours.[97] The research on using TH to treat TBI is of interest to ICH, as there is bleeding and parallel mechanisms of injury (e.g., elevated ICP) in certain cases of TBI. Unfortunately, the recent clinical literature has not been promising. An early clinical trial failed to find benefit at 6 months with the exception of one subgroup that had early initiation of TH and surgical removal of the hematoma.[91,129] Some evidence has suggested that TH may be a useful treatment for cases of TBI with high ICP.[91] To further investigate this effect on ICP the Eurotherm3235 clinical trial studied the impact of TH in TBI patients with high ICP (> 20 mm Hg).[130] Initial investigation found that not only was there no difference in ICP but there was also an increase in unfavourable outcomes in the treated group compared to control group receiving clinical management.[130] Another large randomized controlled trial evaluated early initiation of TH in those with severe TBI but did not find functional benefit at 6 months.[104] The heterogeneity of TBI may make it difficult to find an adequate treatment that suits all patients and targeting specific subgroups may lead to more successful translation.[129] As well, as previously mentioned the duration of TH and rewarming may impact the effectiveness of the treatment in TBI patients.[125]

There is abundant preclinical literature on using TH to treat ischemia with largely positive data on neuroprotective effects when duration and depth of cooling are taken into account.[102,116] Early clinical trials found cooling to be safe and feasible in treating large ischemic strokes and able to reduce elevated ICP.[96,131] These early pilots also noted that the rate of rewarming could have an impact on rebound ICP and related mortality with slower rewarming being more protective than passive rewarming. A larger randomized study, ICTuS-L, evaluated a combination of TH with intravenous thrombolysis vs. intravenous thrombolysis alone.[112] The

combination therapy proved to be safe and feasible but the sample size was too small to determine efficacy[112] and a phase III trial was initiated using the same protocol with the exception of including chilled saline infusion to quicken the time to reach target temperature.[113] Unfortunately this trial, ICTuS-2, was unable to reach adequate recruitment numbers before expiration of funding and the sample size was too small to determine efficacy.[113] As well, the best medical practice for treating ischemic stroke was changed with the development of intra-arterial neurothrombectomy. While unable to determine efficacy, authors found their cooling protocol to be feasible. Another phase III clinical trial in Europe, EuroHYP-1, was also initiated to evaluate combination of TH and best medical practice vs. best medical practice alone but this trial was terminated prior to reaching full recruitment.[132] While the results from clinical trials for ischemic stroke are unclear, it should be noted this is due to lack of power to detect efficacy, not due to mixed and conflicting results. As well, pilot studies found TH to be useful for mitigating elevated ICP, which may be particularly beneficial for patients with large ischemic stroke.

There have been several small-scale trials evaluating the use of TH to treat ICH as well as two larger randomized clinical trials that are currently underway. During the 1950's Howell and colleagues[93] carried out a series of case studies evaluating TH for ICH. While the results they observed were not overly positive with only 2 out of 8 patients surviving, the authors were still optimistic that TH could be used to treat elevated ICP. Since then, only a small handful of human ICH studies have been conducted. In a small study of prolonged, pharmacologically-induced TH, Dohi and others[133] found that in hemorrhagic stroke (i.e., both ICH and SAH combined) TH can effectively lower markers of inflammation early after the stroke. However, this effect was only transient and there were no differences by 4 days post-stroke. Of note, the results of both the ICH and SAH patients were combined and the effects on ICH alone, which has a different

pathophysiology than SAH (i.e., lack of vasospasms), were not clear. More encouragingly, another small study involving only ICH, found that in patients with a substantial hematoma volume (~56 mL) TH applied for 8-10 days could prevent the steep and profound rise in edema seen in historic controls with comparable hematoma volumes.[105] The authors of this study also found that those treated with TH had better functional outcome and lower mortality at 3 months and 1 year post-stroke. Unfortunately, this was not a randomized study (i.e., comparison to historic controls) and involved a small sample size. Still the results of this trial are promising.

A couple of small ICH trials have evaluated the use of TH in combination with another therapy with group sizes ranging from 25-38 per group. Bi and others[134] found that in patients with a large hemorrhage, TH in combination with minimally invasive evacuation of the hematoma marginally improved functional outcome and lowered peri-hematoma levels of inflammation compared to the surgery alone. In a study involving a moderate sized hematoma, TH combined with mannitol significantly improved functional outcome up to 3 weeks post-treatment versus mannitol treatment alone.[135] Interestingly, the study also found that at a week post-treatment, patients treated with the combination therapy also had an increase in regional cerebral blood flow in the lesioned area.

The promising data from these small trials have prompted two multisite, randomized controlled trials, one conducted in Europe and one in North America. The Cooling in Intracerebral Hemorrhage (CINCH) trial is enrolling 50 patients with large hemorrhages from Germany and Austria who will be randomized to an 8 day TH treatment or medical management treatment.[136] At 8 and 11 days post-ICH, hematoma volume and perihematoma edema will be measured and mortality and/or functional outcome will be assessed at 30, 90, and 180 days. In the United States, the Targeted Temperature Management after Intracerebral Hemorrhage (TTM-ICH) trial

is enrolling 50 patients with a minimum baseline hematoma size of 15 mL and patients will receive either 72 hours of TH or maintained normothermia.[137] Neurological deterioration and mortality over the first week will be evaluated as well as functional outcome at discharge and 90 days. Hematoma volume and edema will also be evaluated at 24, 48, 72, and 168 hours. As described in this section, there has been promising clinical research in other modalities of nervous system injury suggesting there is hope that TH can be used to treat ICH, a brain injury that currently has no neuroprotective therapies. However, as outlined in the following section the preclinical data is not clear that TH is a suitable neuroprotective therapy for ICH.

1.4.3 Preclinical Studies of Intracerebral Hemorrhage

Although there is abundant research on using TH to treat experimental ischemic stroke, there is less research on using TH to treat ICH in animals. Preclinical studies of ischemic stroke are generally positive; however, the studies in ICH are less robust particularly when evaluating neuroprotection and functional improvement.[102] In experimental ICH, several animal studies have found TH to be neuroprotective and/or improve behavioural deficits;[138-141] however, other studies have failed to find neurological or function benefit of TH[44,142-146] or had mixed efficacy results.[147] This intermittent protection is surprising as there are numerous overlapping mechanisms of secondary injury common to both ischemic and hemorrhagic stroke that are mitigated by TH (e.g., inflammation, BBB damage, etc.). Within our own lab there has been inconsistent protection, even when matching for ICH models and controlling for treatment parameters such as depth of cooling, time to initiation, duration and rate of rewarming. For example, MacLellan and colleagues[147] found neuroprotection and functional benefit a month post-ICH when systemic hypothermia was delayed for 12 hours. When they used a similar

protocol to evaluate combined use of rehabilitation and TH vs. each treatment alone they failed to find a protective effect on either tissue loss or behaviour, although there were differences such as the size of the lesion and the model of ICH.[148] Thus, more studies are needed to better understand the influence of TH on ICH-induced injury to better understand the inconsistent neuroprotection that can occur even by the same authors.

While the data on efficacy of TH after ICH has been mixed there have been a number of consistent effects. Edema is the most commonly used endpoint in ICH preclinical studies, although its ability to translate to long term neuroprotection and functional benefit is unclear.[76] It has been well established by different investigators that TH can mitigate edema in several ICH animal studies.[44,138-142,146,149,150] However, not all studies have found a reduction in edema despite finding benefit on other measures such as tissue loss and ICP.[126,147] Interestingly, John and Colbourne[126] found that rewarming may have an impact. While they failed to find a beneficial effect of local TH on large hemorrhages, they did find that in animals who underwent fast rewarming had greater levels of edema than treated animals who had slow rewarming. The lack of benefit on edema even with slow rewarming in this study may be due to the late initiation of TH (24 hours post-ICH), which is necessary to avoid bleeding complications in animals who undergo large hemorrhages induced by collagenase. When directly compared, some studies have found early initiation of TH lowered edema while late induction did not.[44,142]

Perhaps unsurprisingly, a number of other effects of TH on mechanisms of secondary damage that may explain TH's fairly robust effect on edema such as inflammation and BBB disruption. Several studies have found that TH reduced inflammation, whether it is number of cells or other markers of activation such as increases in cytokines.[44,139,141,146,150] In fact, MacLellan and others[44] found TH to reduce the number of inflammatory cells by approximately

50%. As well, matrix metalloproteinase 9, which can increase BBB disruption, is mitigated by TH as there is a reduction in the levels of the protein as well as the number of matrix metalloproteinase 9 positive cells.[138,141] Blood brain barrier disruption has been consistently mitigated by TH in many ICH preclinical studies.[44,138-141,146] It should be noted in the majority of these studies that also evaluated efficacy and found benefits, they looked early after ICH (i.e., within 3 days).[138-141] Those that looked later (i.e., a week or more post-ICH) and found improvements in edema, inflammation and/or BBB dysfunction, failed to find neuroprotection and/or behavioural benefit.[44,142,146]

Cooling has been studied for its effects on other mechanisms of injury albeit not to the same extent as neuroprotection, functional benefits, edema, inflammation and BBB disruption. For example, Kawanishi and others[139] found that when TH is delayed 6 hours it significantly reduced DNA oxidative damage. Others have found TH to have a protective effect by upregulating proteins involved in anti-apoptotic pathways, including Bcl-2.[138,140,141] Klahr and others[144] evaluated localized TH on seizure activity but unfortunately did not find that TH reduced the incidence of seizures, the number of animals that experienced seizures or the duration of seizures. As previously discussed in section 1.3.2, excessively high levels of ICP can be a major and sometimes fatal consequence of ICH in humans. Additionally, as discussed in section 1.4.2, clinical research for ischemic stroke, TBI, and ICH has been interested in using TH to mitigate elevated ICP. A preclinical study in rats given a large ICH, found that localized TH alleviated both the peak ICP and average ICP over a 4 day period after ICH in large hemorrhages.[126] Interestingly, this did not lead to a reduction in edema.

Differences in treatment protocol, such as method of inducing treatment, delay in initiating treatment and duration, may be a factor in the conflicting results in preclinical

research[102] but does not fully explain lack of benefit when the same protocol is used in different studies with conflicting results.[44,147] We established our cooling protocol based in previous studies,[44,100,147] as well as, what has been successfully used in the clinic for other conditions such as cardiac arrest.[89] Benefit of TH has been found using the same method of induction, depth and delay in other studies that we used in this thesis.[147] In this thesis we used a well established method of systemic hypothermia in awake and freely moving animals that could effectively control temperature.[145,151] As well, we chose the temperature of 33°C in this thesis as it has been commonly used in our laboratory in previous studies of stroke and found to be tolerable and effective.[100,147] A delay of 12 hours was used in the collagenase-induced ICH experiments as this has been shown to be safe and effective.[147] A delay of 1 hour post-infusion of either thrombin or $FeCl_2$ was used in those experiments as there were no concerns of worsened bleeding and cooling would be initiated while these mechanisms were having an impact on brain tissue. While the rewarming rate used was different, we decided to use a more conservative approach of 0.5°C/hour versus 2°C/hour up to 35°C for 24 hours and then maintained normothermia.[147] Another possibility for conflicting results between studies is due to the model of ICH chosen. We chose to evaluate the use of TH on the collagenase model as there is greater on-going damage, BBB disruption and behavioural deficits and slower hematoma resolution[80] allowing for TH to potentially have a greater impact on hematoma resolution.

1.5 Iron Chelators

As previously discussed, iron has been widely studied as a key contributor to secondary injury following ICH largely due to the influx and breakdown of erythrocytes. Thus, iron chelators have been widely studied as a potential pharmacological treatment for ICH. By binding

iron, this should mitigate the iron-toxicity and oxidative stress after an ICH; however as discussed below, the evidence for using iron chelators to improve recovery after ICH is unclear.

Deferoxamine has been the most widely studied iron chelator and is already currently being used to treat acute iron toxicity outside of the brain.[152] Several studies have found deferoxamine to reduce the number of iron positive cells, edema, oxidative damage, cell death and functional outcome following whole-blood induced ICH.[153-155] However, Warkentin and others[156] did not find deferoxamine to be effective at treating collagenase-induced ICH with no effect on behavioural impairments both in the short and long term or effects on tissue loss and edema. As well, Auriat and others[59] found that while deferoxamine lowered the amount of iron in the brain following collagenase-induced ICH, there was no effect on lesion volume or functional impairment. Wu and colleague[157] did find functional benefit 3 days after collagenase-induced ICH as well as a reduction in number of iron deposits, neuronal degeneration and reactive oxygen species but no effect on lesion volume, edema or hemispheric swelling. Deferoxamine did move on to clinical trial and passed safety trials for ICH patients.[158] Unfortunately, a phase-II clinical trial failed to find benefit.[159] As our lab was previously unable to find neuroprotective or functional benefit with deferoxamine[59,156] and at the time the phase-II clinical trial results were not released we decided to focus our attention on another iron chelator that has been studied for ICH, bipyridine.

Both deferoxamine and bipyridine are bind iron ions; however, there are a couple of differences between the two. For instance, deferoxamine chelates ferric ions while bipyridine chelates ferrous ions[160,161] and it's the ferrous ions that are more volatile and reacts with H_2O_2 to create the hydroxyl radical. Bipyridine is also an intracellular chelator which may allow for sequestering iron ions prior to their release into extracellular space.[162,163] As well, bipyridine is

capable of sequestering of iron ions from transferrin which may promote more stable, long term iron chelation than is capable from transferrin.[163]

The amount of literature on bipyridine is not as substantial as the literature on deferoxamine for hemorrhagic stroke; however, there are a few studies that have found benefit. In animal models of subarachnoid hemorrhage bipyridine treated animals had significantly reduced vasospasm[160] as well as improved functional outcome.[164] In cultured neurons, astrocyte and oligodendrocytes exposed to $FeCl_2$, bipyridine reduced the accumulation of intracellular iron levels whereas deferoxamine did not.[162] Nakamura and others[85] found that treatment with bipyridine after whole-blood ICH in rats qualitatively reduced or eliminated protein oxidation and DNA damage. Another group did a more thorough evaluation of bipyridine for treating ICH injury.[165] This study evaluated both pre- and post-treatment of bipyridine after both collagenase and whole-blood induced ICH in older mice. Pre-treatment of bipyridine did not affect collagenase-induced bleeding, astrocyte activation or neutrophil infiltration but did reduced the number of iron positive cells, number of degenerating neurons, number of activated microglia, generation of reactive oxygen species and damage to white matter.[165] Post-treatment after collagenase induced ICH reduced edema and lesion volume at 3 days post-ICH as well as neurological deficits at 3 and 28 days. Finally, post-treatment with bipyridine following whole-blood ICH led to a reduction in brain water content (i.e., edema) and neurological deficits at 3 days post-ICH.[165] Based on the success seen in subarachnoid hemorrhage and other studies for ICH we chose to evaluate bipyridine with the hopes of using it in combination with TH in future studies.

1.6 General Hypothesis

In this thesis, experiments were conducted to evaluate treatments for hemorrhagic stoke with a focus on therapeutic hypothermia (TH). We specifically wanted to examine the effects of TH on the different mechanisms of injury after hemorrhagic stroke to determine the limitations of using TH to treat this type of stroke. As was previously discussed in this chapter, the data investigating the use of TH as a neuroprotectant against ICH is not strong in comparison to other types of brain injuries. Our first two hypotheses set out to examine the effect of TH on mechanisms of secondary injury after ICH. First, we hypothesized that TH was not adequately targeting two key mechanisms of secondary ICH injury, specifically thrombin and iron- induced injury. And secondly, due to the anti-inflammatory effect of TH, this is hindering endogenous mechanisms of hematoma containment and resolution, and these sides effects negate the ability to detect the protective effects that TH may convey. In addition, we also evaluated an iron chelator, bipyridine, to determine whether this was a suitable pharmacological therapy that could be used in combination with TH to create a strong, well-rounded therapy for hemorrhagic stroke. Our hope was to find a suitable treatment that targeted a mechanism of injury that TH did not, that could be combined with TH and evaluated. However, it is important to note that this pharmacological intervention would need to show its own form of benefit. Our third hypothesis was that if bipyridine provided protection against ICH injury on its own it would be a suitable candidate for investigation as a combination therapy with TH.

1.7 References

1. Heart and Stroke Foundation. Stroke in Canada[Internet]. Ottawa: Heart and Stroke Foundation, (2018, cited 2020 April 27); Available from: https://www.heartandstroke.ca/what-we-do/media-centre/news-releases/canadian-led-international-study-shows-huge-costs-of-delayed-access-to-stroke-care.

2. Bhalla A, Wang Y, Rudd A, Wolfe C. Differences in outcome and predictors between ischemic and intracerebral hemorrhage: The south london stroke register. Stroke 2013 Aug;44(8):2174-2181.

3. Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB. The stroke data bank: Design, methods, and baseline characteristics. Stroke 1988 May;19(5):547-554.

4. Broderick JP, Brott TG, Duldner JE, Tomsick T, Huster G. Volume of intracerebral hemorrhage. A powerful and easy-to-use predictor of 30-day mortality. Stroke 1993 Jul;24(7):987-993.

5. Navarrete-Navarro P, Hart WM, Lopez-Bastida J, Christensen MC. The societal costs of intracerebral hemorrhage in spain. European Journal of Neurology 2007 May;14(5):556-562.

6. Hemphill J, Greenberg S, Anderson C, Becker K, Bendok B, Cushman M, et al. Guidelines for the management of spontaneous intracerebral hemorrhage: A guideline for healthcare professionals from the american heart association/american stroke association. Stroke 2015 Jul;46(7):2032-2060.

7. Howard G, Cushman M, Howard VJ, Kissela BM, Kleindorfer DO, Moy CS, et al. Risk factors for intracerebral hemorrhage: The REasons for geographic and racial differences in stroke (REGARDS) study. Stroke 2013 May;44(5):1282-1287.

8. Martini SR, Flaherty ML, Brown WM, Haverbusch M, Comeau ME, Sauerbeck LR, et al. Risk factors for intracerebral hemorrhage differ according to hemorrhage location. Neurology 2012 Dec 4,;79(23):2275-2282.

9. Vonsattel JPG, Myers RH, Tessa Hedley-Whyte E, Ropper AH, Bird ED, Richardson EP. Cerebral amyloid angiopathy without and with cerebral hemorrhages: A comparative histological study. Annals of Neurology 1991 Nov;30(5):637-649.

10. Cavallini A, Fanucchi S, Persico A. Warfarin-associated intracerebral hemorrhage. Neurol Sci 2008 Sep;29(S2):266-268.

11. Dowlatshahi D, Butcher KS, Asdaghi N, Nahirniak S, Bernbaum ML, Giulivi A, et al. Poor prognosis in warfarin-associated intracranial hemorrhage despite anticoagulation reversal. Stroke 2012 Jul;43(7):1812-1817.

12. Flibotte JJ, Hagan N, O'Donnell J, Greenberg SM, Rosand J. Warfarin, hematoma expansion, and outcome of intracerebral hemorrhage. Neurology 2004 Sep 28,;63(6):1059-1064.

13. Brott T, Broderick J, Kothari R, Barsan W, Tomsick T, Sauerbeck L, et al. Early hemorrhage growth in patients with intracerebral hemorrhage. Stroke 1997 Jan;28(1):1-5.

14. Davis SM, Broderick J, Hennerici M, Brun NC, Diringer MN, Mayer SA, et al. Hematoma growth is a determinant of mortality and poor outcome after intracerebral hemorrhage. Neurology 2006 Apr 25,;66(8):1175-1181.

15. Wada R, Aviv RI, Fox AJ, Sahlas DJ, Gladstone DJ, Tomlinson G, et al. CT angiography "spot sign" predicts hematoma expansion in acute intracerebral hemorrhage. Stroke 2007 Apr;38(4):1257-1262.

16. Mayer SA, Brun NC, Begtrup K, Broderick J, Davis S, Diringer MN, et al. Efficacy and safety of recombinant activated factor VII for acute intracerebral hemorrhage. N Engl J Med 2008 May 15,;358(20):2127-2137.

17. Rincon F, Rincon F, Lyden P, Lyden P, Mayer S, Mayer S. Relationship between temperature, hematoma growth, and functional outcome after intracerebral hemorrhage. Neurocrit Care 2013 Feb;18(1):45-53.

18. MacLellan CL, Colbourne F. Mild to moderate hyperthermia does not worsen outcome after severe intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2005 August 1,;25(8):1020-1029.

19. Penner M, Silasi G, Wowk S, Warkentin L, Colbourne F. Brief hyperthermia does not worsen outcome after striatal hemorrhage in rats. Curr Neurovasc Res 2011 Feb;8(1):35-43.

20. Dandapani BK, Suzuki S, Kelley RE, Reyes-Iglesias Y, Duncan RC. Relation between blood pressure and outcome in intracerebral hemorrhage. Stroke 1995 Jan;26(1):21-24.

21. Anderson CS, Heeley E, Huang Y, Wang J, Wang J, Stapf C, et al. Rapid blood-pressure lowering in patients with acute intracerebral hemorrhage. The New England Journal of Medicine 2013 Jun 20,;368(25):2355-2365.

22. Qureshi AI, Palesch YY, Barsan WG, Hanley DF, Hsu CY, Martin RL, et al. Intensive blood-pressure lowering in patients with acute cerebral hemorrhage. The New England Journal of Medicine 2016 Sep 15,;375(11):1033-1043.

23. Leasure AC, Qureshi AI, Murthy SB, Kamel H, Goldstein JN, Woo D, et al. Association of intensive blood pressure reduction with risk of hematoma expansion in patients with deep intracerebral hemorrhage. JAMA neurology 2019 May 13,;76(8):949.

24. Menon RS, Burgess RE, Wing JJ, Gibbons MC, Shara NM, Fernandez S, et al. Predictors of highly prevalent brain ischemia in intracerebral hemorrhage: High prevalence of ischemic infarcts in ich. Annals of Neurology 2012 Feb 1,;71(2):199-205.

25. Gioia L, Kate M, Choi V, Sivakumar L, Jeerakathil T, Kosior J, et al. Ischemia in intracerebral hemorrhage is associated with leukoaraiosis and hematoma volume, not blood pressure reduction. Stroke 2015 Jun;46(6):1541-1547.

26. Prabhakaran S, Naidech AM. Ischemic brain injury after intracerebral hemorrhage: A critical review. Stroke 2012 Aug;43(8):2258-2263.

27. Butcher KS, Jeerakathil T, Hill M, Demchuk AM, Dowlatshahi D, Coutts SB, et al. The intracerebral hemorrhage acutely decreasing arterial pressure trial. Stroke 2013 Mar;44(3):620-626.

28. Gioia L, Klahr A, Kate M, Buck B, Dowlatshahi D, Jeerakathil T, et al. The intracerebral hemorrhage acutely decreasing arterial pressure trial II (ICH ADAPT II) protocol. BMC neurology 2017 May 19,;17(1):100.

29. Goldstein JN, Thomas SH, Frontiero V, Joseph A, Engel C, Snider R, et al. Timing of fresh frozen plasma administration and rapid correction of coagulopathy in warfarin-related intracerebral hemorrhage. Stroke 2006 Jan 1,;37(1):151.

30. Hinchey JA, Shephard T, Furie K, Smith D, Wang D, Tonn S, et al. Formal dysphagia screening protocols prevent pneumonia. Stroke 2005 Sep 1,;36(9):1972-1976.

31. Ko S, Choi HA, Parikh G, Helbok R, Schmidt JM, Lee K, et al. Multimodality monitoring for cerebral perfusion pressure optimization in comatose patients with intracerebral hemorrhage. Stroke 2011 Nov;42(11):3087-3092.

32. Keep RF, Xi G, Hua Y, Xiang J. Clot formation, vascular repair and hematoma resolution after ICH, a coordinating role for thrombin? Acta neurochirurgica. Supplement 2011;111:71-75.

33. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

34. Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. Prog Neurobiol 2010 Dec;92(4):463-477.

35. Caliaperumal J, Brodie S, Ma Y, Colbourne F. Thrombin causes neuronal atrophy and acute but not chronic cell death. Can J Neurol Sci 2014 Nov;41(6):714-720.

36. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

37. Power C, Henry S, Del Bigio MR, Larsen PH, Corbett D, Imai Y, et al. Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases. Annals of Neurology 2003 Jun;53(6):731-742.

38. Lee KR, Kawai N, Kim S, Sagher O, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: Effects of thrombin on cerebral blood flow, blood-brain barrier permeability, and cell survival in a rat model. Journal of neurosurgery 1997 Feb;86(2):272.

39. Yang S, Song S, Hua Y, Nakamura T, Keep RF, Xi G. Effects of thrombin on neurogenesis after intracerebral hemorrhage. Stroke 2008 Jul;39(7):2079-2084.

40. Yang A-, Zhou H, Lin Y, Luo J, Cui H, Tang T, et al. Thrombin promotes the expression of thrombospondin-1 and -2 in a rat model of intracerebral hemorrhage. J Neurol Sci 2012 Dec 15,;323(1-2):141-146.

41. Auriat AM, Wowk S, Colbourne F. Rehabilitation after intracerebral hemorrhage in rats improves recovery with enhanced dendritic complexity but no effect on cell proliferation. Behav Brain Res 2010 Dec 06,;214(1):42-47.

42. Hua Y, Keep RF, Gu Y, Xi G. Thrombin and brain recovery after intracerebral hemorrhage. Stroke 2009 Mar;40(3 Suppl):88.

43. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

44. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

45. Del Bigio MR, Yan HJ, Buist R, Peeling J. Experimental intracerebral hemorrhage in rats. magnetic resonance imaging and histopathological correlates. Stroke 1996 Dec;27(12):2312-2320.

46. Wasserman JK, Schlichter LC. Neuron death and inflammation in a rat model of intracerebral hemorrhage: Effects of delayed minocycline treatment. Brain Research 2007;1136(1):208-218.

47. Wasserman JK, Zhu X, Schlichter LC. Evolution of the inflammatory response in the brain following intracerebral hemorrhage and effects of delayed minocycline treatment. Brain Res 2007 Nov 14,;1180:140-154.

48. Xue M, Balasubramaniam J, Buist RJ, Peeling J, Del Bigio MR. Periventricular/intraventricular hemorrhage in neonatal mouse cerebrum. J Neuropathol Exp Neurol 2003 Nov;62(11):1154-1165.

49. Wu J, Yang S, Hua Y, Liu W, Keep RF, Xi G. Minocycline attenuates brain edema, brain atrophy and neurological deficits after intracerebral hemorrhage. Acta Neurochir Suppl 2010;106:147-150.

50. Zhao X, Sun G, Zhang J, Strong R, Song W, Gonzales N, et al. Hematoma resolution as a target for intracerebral hemorrhage treatment: Role for peroxisome proliferator-activated receptor gamma in microglia/macrophages. Ann Neurol 2007 Apr;61(4):352-362.

51. Zhao X, Grotta J, Gonzales N, Aronowski J. Hematoma resolution as a therapeutic target: The role of microglia/macrophages. Stroke 2009 Mar 1,;40(3, Supplement 1):S92-S94.

52. Zhao X, Sun G, Ting S, Song S, Zhang J, Edwards NJ, et al. Cleaning up after ICH: The role of Nrf2 in modulating microglia function and hematoma clearance. J Neurochem 2015 Apr;133(1):144-152.

53. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: Secondary brain injury. Stroke 2011 Jun;42(6):1781-1786.

54. Wagner KR, Sharp FR, Ardizzone TD, Lu A, Clark JF. Heme and iron metabolism: Role in cerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2003 Jun;23(6):629-652.

55. Hahl P, Davis T, Washburn C, Rogers JT, Smith A. Mechanisms of neuroprotection by hemopexin: Modeling the control of heme and iron homeostasis in brain neurons in inflammatory states. J Neurochem 2013 Apr;125(1):89-101.

56. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke 2003 Dec 1,;34(12):2964-2969.

57. Willmore LJ, Rubin JJ. Formation of malonaldehyde and focal brain edema induced by subpial injection of FeCl2 into rat isocortex. Brain research 1982 Aug 19,;246(1):113-119.

58. Wu J, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. Brain Res 2002 Oct 25,;953(1-2):45-52.

59. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

60. Katsu M, Niizuma K, Yoshioka H, Okami N, Sakata H, Chan PH. Hemoglobin-induced oxidative stress contributes to matrix metalloproteinase activation and blood-brain barrier dysfunction in vivo. J Cereb Blood Flow Metab 2010 Dec;30(12):1939-1950.

61. Nakamura T, Keep RF, Hua Y, Hoff JT, Xi G. Oxidative DNA injury after experimental intracerebral hemorrhage. Brain Res 2005 Mar 28,;1039(1-2):30-36.

62. Willmore LJ, Ballinger WE, Boggs W, Sypert GW, Rubin JJ. Dendritic alterations in rat isocortex within an iron-induced chronic epileptic focus. Neurosurgery 1980 Aug;7(2):142-146.

63. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

64. Hua Y, Nakamura T, Keep RF, Wu J, Schallert T, Hoff JT, et al. Long-term effects of experimental intracerebral hemorrhage: The role of iron. J Neurosurg 2006 Feb;104(2):305-312.

65. Kell DB. Towards a unifying, systems biology understanding of large-scale cellular death and destruction caused by poorly liganded iron: Parkinson's, huntington's, alzheimer's, prions,

bactericides, chemical toxicology and others as examples. Arch Toxicol 2010 Nov;84(11):825-889.

66. Zhao X, Song S, Sun G, Strong R, Zhang J, Grotta JC, et al. Neuroprotective role of haptoglobin after intracerebral hemorrhage. J Neurosci 2009 Dec 16,;29(50):15819-15827.

67. Kell DB. Iron behaving badly: Inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC Med Genomics 2009 Jan 08,;2:2.

68. Wu G, Xi G, Hua Y, Sagher O. T2* magnetic resonance imaging sequences reflect brain tissue iron deposition following intracerebral hemorrhage. Transl Stroke Res 2010 Mar 01,;1(1):31-34.

69. Chen M, Awe OO, Chen-Roetling J, Regan RF. Iron regulatory protein-2 knockout increases perihematomal ferritin expression and cell viability after intracerebral hemorrhage. Brain Res 2010 -6-14;1337:95-103.

70. Grienberger HJ, Pillai DR, Schlachetzki F, Gruber M, Dittmar MS. Detection of free radicals by isolated perfusion of the rat brain following hemorrhagic stroke: A novel approach to cerebrovascular biomarker research. Exp Brain Res 2010 Oct;206(3):311-317.

71. 韩宁, Han N, Ding S, 丁素菊, Wu T, 吴涛, et al. Correlation of free radical level and apoptosis after intracerebral hemorrhage in rats. Neurosci Bull 2008 Dec;24(6):351-358.

72. Wang G, Yang Q, Li G, Wang L, Hu W, Tang Q, et al. Time course of heme oxygenase-1 and oxidative stress after experimental intracerebral hemorrhage. Acta Neurochir (Wien) 2011 Feb;153(2):319-325.

73. Papacocea T, Papacocea R, Badarau A, Ion AD, Burga I, Gaman L, et al. Oxidative stress and antioxidant therapy in intracerebral hemorrhage. Therapeutics, Pharmacology and Clinical Toxicology 2011 December;15(4):270-273.

74. Lyden PD, Shuaib A, Lees KR, Davalos A, Davis SM, Diener H, et al. Safety and tolerability of NXY-059 for acute intracerebral hemorrhage: The CHANT trial. Stroke 2007 Aug 1,;38(8):2262-2269.

75. Zafar A, Khan FS. Clinical and radiological features of intracerebral haemorrhage in hypertensive patients. J Pak Med Assoc 2008 Jul;58(7):356-358.

76. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

77. Bullock R, Mendelow AD, Teasdale GM, Graham DI. Intracranial haemorrhage induced at arterial pressure in the rat. part 1: Description of technique, ICP changes and neuropathological findings. Neurol Res 1984 Dec;6(4):184-188.

78. Rosenberg GA, Mun-Bryce S, Wesley M, Kornfeld M. Collagenase-induced intracerebral hemorrhage in rats. Stroke 1990 May;21(5):801-807.

79. Hiploylee C, Colbourne F. Intracranial pressure measured in freely moving rats for days after intracerebral hemorrhage. Exp Neurol 2014 May;255:49-55.

80. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

81. Klahr AC, Dickson CT, Colbourne F. Seizure activity occurs in the collagenase but not the blood infusion model of striatal hemorrhagic stroke in rats. Transl Stroke Res 2015 Feb;6(1):29-38.

82. Smeda JS. Hemorrhagic stroke development in spontaneously hypertensive rats fed a north american, japanese-style diet. Stroke 1989 Sep;20(9):1212-1218.

83. Lee J, Zhai G, Liu Q, Gonzales ER, Yin K, Yan P, et al. Vascular permeability precedes spontaneous intracerebral hemorrhage in stroke-prone spontaneously hypertensive rats. Stroke 2007 Dec;38(12):3289-3291.

84. Lee KR, Drury I, Vitarbo E, Hoff JT. Seizures induced by intracerebral injection of thrombin: A model of intracerebral hemorrhage. Journal of neurosurgery 1997 Jul;87(1):73-78.

85. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

86. Buchan A, Pulsinelli WA. Hypothermia but not the N-methyl-D-aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia. J Neurosci 1990 Jan;10(1):311-316.

87. Corbett D, Evans S, Thomas C, Wang D, Jonas RA. MK-801 reduced cerebral ischemic injury by inducing hypothermia. Brain Res 1990 Apr 30,;514(2):300-304.

88. Peliowski-Davidovich A. Hypothermia for newborns with hypoxic ischemic encephalopathy. Paediatrics & child health 2012 Jan;17(1):41-43.

89. Howes D, Gray SH, Brooks SC, Boyd JG, Djogovic D, Golan E, et al. Canadian guidelines for the use of targeted temperature management (therapeutic hypothermia) after cardiac arrest: A joint statement from the canadian critical care society (CCCS), canadian neurocritical care society (CNCCS), and the canadian critical care trials group (CCCTG). Resuscitation 2016 Jan;98:48-63.

90. González-Ibarra FP, Varon J, López-Meza EG. Therapeutic hypothermia: Critical review of the molecular mechanisms of action. Front Neurol 2011;2:4.

91. Karnatovskaia LV, Wartenberg KE, Freeman WD. Therapeutic hypothermia for neuroprotection: History, mechanisms, risks, and clinical applications. Neurohospitalist 2014 Jul;4(3):153-163.

92. Thomson J. Report of a case of sanguineous apoplexy successfully treated. The Lancet 1848 June /06;51(1293):634-635.

93. Howell DA, Posnikoff J, Stratford JG. Prolonged hypothermia in treatment of massive cerebral haemorrhage; a preliminary report. Canadian Medical Association journal 1956 Sep 1,;75(5):388-394.

94. The Hypothermia after Cardiac Arrest Study Group. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. The New England Journal of Medicine 2002 Feb 21,;346(8):549-556.

95. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med 2005 Oct 13,;353(15):1574-1584.

96. Schwab S, Schwarz S, Spranger M, Keller E, Bertram M, Hacke W. Moderate hypothermia in the treatment of patients with severe middle cerebral artery infarction. Stroke 1998 Dec;29(12):2461-2466.

97. The Brain Trauma Foundation. Guidelines for the management of severe traumatic brain injury. Journal of Neurotrauma 2007;24.

98. Colbourne F, Corbett D. Delayed postischemic hypothermia: A six month survival study using behavioral and histological assessments of neuroprotection. J Neurosci 1995 Nov;15(11):7250-7260.

99. Colbourne F, Li H, Buchan AM. Indefatigable CA1 sector neuroprotection with mild hypothermia induced 6 hours after severe forebrain ischemia in rats. J Cereb Blood Flow Metab 1999 Jul;19(7):742-749.

100. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

101. Colbourne F, Corbett D, Zhao Z, Yang J, Buchan AM. Prolonged but delayed postischemic hypothermia: A long-term outcome study in the rat middle cerebral artery occlusion model. J Cereb Blood Flow Metab 2000 Dec;20(12):1702-1708.

102. MacLellan CL, Clark DL, Silasi G, Colbourne F. Use of prolonged hypothermia to treat ischemic and hemorrhagic stroke. J Neurotrauma 2009 Mar;26(3):313-323.

103. Clark DL, Penner M, Wowk S, Orellana-Jordan I, Colbourne F. Treatments (12 and 48 h) with systemic and brain-selective hypothermia techniques after permanent focal cerebral ischemia in rat. Exp Neurol 2009 Dec;220(2):391-399.

104. Cooper DJ, Nichol AD, Bailey M, Bernard S, Cameron PA, Pili-Floury S, et al. Effect of early sustained prophylactic hypothermia on neurologic outcomes among patients with severe traumatic brain injury: The POLAR randomized clinical trial. JAMA 2018 12 04,;320(21):2211-2220.

105. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

106. Dididze M, Green BA, Dietrich WD, Vanni S, Wang MY, Levi AD. Systemic hypothermia in acute cervical spinal cord injury: A case-controlled study. Spinal cord 2013 May;51(5):395-400.

107. Abou-Chebl A, DeGeorgia MA, Andrefsky JC, Krieger DW. Technical refinements and drawbacks of a surface cooling technique for the treatment of severe acute ischemic stroke. Neurocrit Care 2004;1(2):131-143.

108. Georgiadis D, Schwarz S, Kollmar R, Schwab S. Endovascular cooling for moderate hypothermia in patients with acute stroke: First results of a novel approach. Stroke 2001 Nov;32(11):2550-2553.

109. Busch H-, Eichwede F, Födisch M, Taccone FS, Wöbker G, Schwab T, et al. Safety and feasibility of nasopharyngeal evaporative cooling in the emergency department setting in survivors of cardiac arrest. Resuscitation 2010 Aug;81(8):943-949.

110. Bernard SA, Smith K, Cameron P, Masci K, Taylor DM, Cooper DJ, et al. Induction of therapeutic hypothermia by paramedics after resuscitation from out-of-hospital ventricular fibrillation cardiac arrest: A randomized controlled trial. Circulation 2010 Aug 17,;122(7):737-742.

111. Cortez E, Panchal AR, Davis J, Zeeb P, Keseg DP. Clinical outcomes in cardiac arrest patients following prehospital treatment with therapeutic hypothermia. Prehosp Disaster Med 2015 Oct;30(5):452-456.

112. Hemmen TM, Raman R, Guluma KZ, Meyer BC, Gomes JA, Cruz-Flores S, et al. Intravenous thrombolysis plus hypothermia for acute treatment of ischemic stroke (ICTuS-L): Final results. Stroke 2010 Oct;41(10):2265-2270.

113. Lyden P, Hemmen T, Grotta J, Rapp K, Ernstrom K, Rzesiewicz T, et al. Results of the ICTuS 2 trial (intravascular cooling in the treatment of stroke 2). Stroke 2016;47(12):2888-2895.

114. NIELSEN N, HOVDENES J, NILSSON F, RUBERTSSON S, STAMMET P, SUNDE K, et al. Outcome, timing and adverse events in therapeutic hypothermia after out-of-hospital cardiac arrest. Acta Anaesthesiologica Scandinavica 2009 Aug;53(7):926-934.

115. Schwab S, Georgiadis D, Berrouschot J, Schellinger PD, Graffagnino C, Mayer SA. Feasibility and safety of moderate hypothermia after massive hemispheric infarction. Stroke 2001 Sep;32(9):2033-2035.

116. Wu T, Grotta JC. Hypothermia for acute ischaemic stroke. Lancet Neurol 2013 Mar;12(3):275-284.

117. Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, et al. Stroke-induced immunodepression: Experimental evidence and clinical relevance. Stroke 2007 Feb;38(2 Suppl):770-773.

118. Shi K, Wood K, Shi F, Wang X, Liu Q. Stroke-induced immunosuppression and poststroke infection. Stroke and Vascular Neurology 2018 Mar;3(1):34-41.

119. Polderman KH. Hypothermia and coagulation. Crit Care 2012 June 16;16(Suppl 2):A20-A20.

120. Joshi B, Brady K, Lee J, Easley B, Panigrahi R, Smielewski P, et al. Impaired autoregulation of cerebral blood flow during rewarming from hypothermic cardiopulmonary bypass and its potential association with stroke. Anesth Analg 2010 -2-01;110(2):321-328.

121. Scaravilli V, Bonacina D, Citerio G. Rewarming: Facts and myths from the systemic perspective. Crit Care 2012;16(Suppl 2):A25.

122. Ueda Y, Suehiro E, Wei EP, Kontos HA, Povlishock JT. Uncomplicated rapid posthypothermic rewarming alters cerebrovascular responsiveness. Stroke 2004 Feb 1,;35(2):601-606.

123. Suehiro E, Povlishock JT. Exacerbation of traumatically induced axonal injury by rapid posthypothermic rewarming and attenuation of axonal change by cyclosporin A. J Neurosurg 2001 Mar;94(3):493-498.

124. Nakamura T, Miyamoto O, Yamagami S, Hayashida Y, Itano T, Nagao S. Influence of rewarming conditions after hypothermia in gerbils with transient forebrain ischemia. J Neurosurg 1999 Jul;91(1):114-120.

125. Jiang J, Xu W, Li W, Gao G, Bao Y, Liang Y, et al. Effect of long-term mild hypothermia or short-term mild hypothermia on outcome of patients with severe traumatic brain injury. Journal of Cerebral Blood Flow & Metabolism 2006 Jun;26(6):771-776.

126. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

127. Madhavan K, Benglis DM, Wang MY, Vanni S, Lebwohl N, Green BA, et al. The use of modest systemic hypothermia after iatrogenic spinal cord injury during surgery. Ther Hypothermia Temp Manag 2012 Dec;2(4):183-192.

128. Ahmad FU, Wang MY, Levi AD. Hypothermia for acute spinal cord Injury—A review. World Neurosurgery 2014 Jul;82(1-2):207-214.

129. Dietrich WD, Bramlett HM. Therapeutic hypothermia and targeted temperature management for traumatic brain injury: Experimental and clinical experience. Brain circulation 2017 Oct;3(4):186-198.

130. Andrews PJD, Sinclair HL, Rodriguez A, Harris BA, Battison CG, Rhodes JKJ, et al. Hypothermia for intracranial hypertension after traumatic brain injury. The New England Journal of Medicine 2015 Dec 17,;373(25):2403-2412.

131. Steiner T, Friede T, Aschoff A, Schellinger PD, Schwab S, Hacke W. Effect and feasibility of controlled rewarming after moderate hypothermia in stroke patients with malignant infarction of the middle cerebral artery. Stroke 2001 Dec 01,;32(12):2833-2835.

132. van der Worp, H. Bart, Macleod MR, Bath PMW, Demotes J, Durand-Zaleski I, Gebhardt B, et al. EuroHYP-1: European multicenter, randomized, phase III clinical trial of therapeutic hypothermia plus best medical treatment vs. best medical treatment alone for acute ischemic stroke. International Journal of Stroke 2014 Jul;9(5):642-645.

133. Dohi K, Jimbo H, Ikeda Y, Fujita S, Ohtaki H, Shioda S, et al. Pharmacological brain cooling with indomethacin in acute hemorrhagic stroke: Antiinflammatory cytokines and antioxidative effects. Acta Neurochir Suppl 2006;96:57-60.

134. BI Y, HUAN Y, CAI W, WANG X, LIANG Z, LIU Z, et al. Mild hypothermia in combination with minimally invasive evacuation of hematoma reduces inflammatory damage in patients via the nuclear factor-κB pathway. Experimental and Therapeutic Medicine 2014 Dec;8(6):1717-1722.

135. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

136. Kollmar R, Juettler E, Huttner HB, Dörfler A, Staykov D, Kallmuenzer B, et al. Cooling in intracerebral hemorrhage (CINCH) trial: Protocol of a randomized german-austrian clinical trial. Int J Stroke 2012 Feb;7(2):168-172.

137. Rincon F, Friedman DP, Bell R, Mayer SA, Bray PF. Targeted temperature management after intracerebral hemorrhage (TTM-ICH): Methodology of a prospective randomized clinical trial. Int J Stroke 2014 Jul;9(5):646-651.

138. Sun H, Tang Y, Li L, Guan X, Wang D. Effects of local hypothermia on neuronal cell apoptosis after intracerebral hemorrhage in rats. J Nutr Health Aging 2015 Mar;19(3):291-298.

139. Kawanishi M, Kawai N, Nakamura T, Luo C, Tamiya T, Nagao S. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. J Stroke Cerebrovasc Dis 2008;17(4):187-195.

140. Wei S, Sun J, Li J, Wang L, Hall CL, Dix TA, et al. Acute and delayed protective effects of pharmacologically induced hypothermia in an intracerebral hemorrhage stroke model of mice. Neuroscience 2013;252:489-500.

141. Zhu Y, Liu C, Sun Z. Early combined therapy with pharmacologically induced hypothermia and edaravone exerts neuroprotective effects in a rat model of intracerebral hemorrhage. Cell Biochem Biophys 2015 Nov;73(2):581-587.

142. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

143. Fingas M, Penner M, Silasi G, Colbourne F. Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia. Experimental Neurology 2009;219(1):156-162.

144. Klahr AC, Dietrich K, Dickson CT, Colbourne F. Prolonged localized mild hypothermia does not affect seizure activity after intracerebral hemorrhage in rats. Ther Hypothermia Temp Manag 2016 Mar;6(1):40-47.

145. MacLellan C, Shuaib A, Colbourne F. Failure of delayed and prolonged hypothermia to favorably affect hemorrhagic stroke in rats. Brain Research 2002;958(1):192-200.

146. Sun H, Tang Y, Guan X, Li L, Wang D. Effects of selective hypothermia on blood-brain barrier integrity and tight junction protein expression levels after intracerebral hemorrhage in rats. Biol Chem 2013 Oct;394(10):1317-1324.

147. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

148. Maclellan CL, Grams J, Adams K, Colbourne F. Combined use of a cytoprotectant and rehabilitation therapy after severe intracerebral hemorrhage in rats. Brain Res 2005 Nov 23,;1063(1):40-47.

149. Kawai N, Nakamura T, Nagao S. Effects of brain hypothermia on brain edema formation after intracerebral hemorrhage in rats. Acta Neurochir Suppl 2002;81:233-235.

150. Wagner KR, Beiler S, Beiler C, Kirkman J, Casey K, Robinson T, et al. Delayed profound local brain hypothermia markedly reduces interleukin-1beta gene expression and vasogenic edema development in a porcine model of intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:177-182.

151. Colbourne F, Sutherland GR, Auer RN. An automated system for regulating brain temperature in awake and freely moving rodents. J Neurosci Methods 1996 Aug;67(2):185-190.

152. Proudfoot AT, Simpson D, Dyson EH. Management of acute iron poisoning. Medical toxicology 1986 Mar;1(2):83-100.

153. Gu Y, Hua Y, Keep RF, Morgenstern LB, Xi G. Deferoxamine reduces intracerebral hematoma-induced iron accumulation and neuronal death in piglets. Stroke 2009 Jun 1,;40(6):2241-2243.

154. Nakamura T, Keep RF, Hua Y, Schallert T, Hoff JT, Xi G. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. Journal of neurosurgery 2004 Apr;100(4):672-678.

155. Okauchi M, Hua Y, Keep RF, Morgenstern LB, Schallert T, Xi G. Deferoxamine treatment for intracerebral hemorrhage in aged rats: Therapeutic time window and optimal duration. Stroke 2010 Feb 1,;41(2):375-382.

156. Warkentin LM, Auriat AM, Wowk S, Colbourne F. Failure of deferoxamine, an iron chelator, to improve outcome after collagenase-induced intracerebral hemorrhage in rats. Brain Research 2009;1309:95-103.

157. Wu H, Wu T, Xu X, Wang J, Wang J. Iron toxicity in mice with collagenase-induced intracerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2011 May;31(5):1243-1250.

158. Selim M, Yeatts S, Goldstein JN, Gomes J, Greenberg S, Morgenstern LB, et al. Safety and tolerability of deferoxamine mesylate in patients with acute intracerebral hemorrhage. Stroke 2011 Nov;42(11):3067-3074.

159. Selim M, Foster LD, Moy CS, Xi G, Hill MD, Morgenstern LB, et al. Deferoxamine mesylate in patients with intracerebral haemorrhage (i-DEF): A multicentre, randomised, placebo-controlled, double-blind phase 2 trial. The Lancet Neurology 2019 May;18(5):428-438.

160. Horky LL, Pluta RM, Boock RJ, Oldfield EH. Role of ferrous iron chelator 2,2′-dipyridyl in preventing delayed vasospasm in a primate model of subarachnoid hemorrhage. Journal of neurosurgery 1998 Feb;88(2):298-303.

161. Ikeda Y, Ikeda K, Long DM. Comparative study of different iron-chelating agents in cold-induced brain edema. Neurosurgery 1989 Jun;24(6):820-824.

162. Kress GJ, Dineley KE, Reynolds IJ. The relationship between intracellular free iron and cell injury in cultured neurons, astrocytes, and oligodendrocytes. Journal of Neuroscience 2002 Jul 15,;22(14):5848-5855.

163. Nunez MT, Cole ES, Glass J. The reticulocyte plasma membrane pathway of iron uptake as determined by the mechanism of alpha, alpha'-dipyridyl inhibition. J Biol Chem 1983 Jan 25,;258(2):1146-1151.

164. Yu YY, Niu L, Gao L, Zhang GL, Li J, Deng JP, et al. Ferrous chelator 2,2-dipyridyl attenuates cerebral vasospasm after experimental subarachnoid haemorrhage in rabbits. The Journal of International Medical Research 2010 Mar;38(2):583-592.

165. Wu H, Wu T, Li M, Wang J. Efficacy of the lipid-soluble iron chelator 2,2'-dipyridyl against hemorrhagic brain injury. Neurobiol Dis 2012 Jan;45(1):388-394.

Chapter Two

Mild Therapeutic Hypothermia Does Not Reduce Thrombin-Induced Brain Injury

2.1 Introduction

Therapeutic hypothermia has been widely studied as a neuroprotectant against traumatic, ischemic, and hemorrhagic brain injuries. As reviewed,[1-3] the clinical successes with cardiac arrest in adults and hypoxic-ischemic injury in neonates were predicted by animal studies, and there is hope that cooling will prove useful for other insults such as stroke. Generally, however, most neuroprotectants have not translated, which is a troubling problem and the subject of much study and speculation.[4-6] From this, it is clear that some inconsistencies result from important individual (e.g., age), insult (e.g., stroke type and severity), and treatment parameters (e.g., dose) that differ among studies. Of course other factors contribute to variability among studies, such as incomplete methodological disclosure, choice of statistical test, and chance. Inconsistencies and failure to replicate experiments are why negative findings, including hypothermia, should be published so that they can be considered in the development process and for aiding in selecting therapies that go forward for clinical study.[7]

Numerous animal studies have assessed whether delayed hypothermia improves outcome in models of global and focal ischemia, usually with positive findings[1,8] that have led to a number of clinical studies.[9-12] In contrast, the data from several animal models of intracerebral hemorrhage (ICH) have been mixed and, in our opinion, less robust.[13-22] Conversely, the few clinical studies with select patients are generally positive[23-25] with more study underway.[26] For instance, Kollmar and others[24] have shown that prolonged mild cooling (e.g., 35°C for 10 days) appears to improve outlook after larger ICHs.[24,25] Although these are not large randomized controlled trials, the findings are encouraging and they suggest that cooling improves outcome by reducing perihematoma edema.

Edema is thought to be an important complication of ICH that leads to elevated intracranial pressure (ICP).[27,28] Not surprisingly, edema is used in approximately half of all rodent ICH neuroprotection studies owing to this and other presumed harmful effects, and, no doubt, due to its ease of measurement.[29] However, not all clinical studies agree on the contribution of edema and ICP to survival, injury, and functional outlook, and there are other determinants, such as hematoma size, that are better predictors across a broad range of patients.[27,28,30] Similarly, we recently questioned the role of edema in the common rat ICH models.[31] We showed that only modest increases in ICP and decreases in cerebral perfusion pressure followed large to very large collagenase-induced ICHs with no notable elevation in ICP in the 100-μL blood infusion model despite having substantially elevated brain water content (BWC) and sizeable hematomas in all models. Accordingly, we argue that reductions in BWC alone, presumably from reducing edema (vs. affecting serum extrusion from the clot), may often not translate into a neuroprotective effect in rodents (or humans) suffering from an ICH. Regardless, our rat ICP findings do not exclude using BWC to predict survival, behavior, and histological outlook (i.e., as a surrogate marker), and it is only logical that extreme levels of BWC are harmful or even lethal but this is not something commonly modeled in rodents (compare the low mortality rates in the rat ICH models with humans). In this light, it is encouraging that many animal hypothermia studies find that delayed cooling lowers BWC after an ICH[14,16,17,20,22] because it is expected that this would translate into improved survival and reduced injury in those patients (and animal models) whose edema and ICP responses are of significant harm. Further, hypothermia should also be helpful by reducing inflammation, blood–brain barrier injury, and other mechanisms of injury.

Given the potential for using cooling to treat ICH,[23-25] our lab began by determining the effects of hypothermia in the two most widely used models—infusing collagenase or whole blood into the striatum of rats.[29,32] We varied treatment parameters, including the duration and method of cooling and intervention delay. Despite multiple studies from one lab, the efficacy results were mixed and we were unable to identify a consistently ‘‘neuroprotective’’ cooling regimen—in other words, one that reduces cell death and improves functional recovery.[14,15,18-20] Thus, we have begun studying whether cooling mitigates behavioral impairments, injury, and edema caused by the known contributors to secondary degeneration after ICH, such as thrombin production and iron release. Thrombin is quickly produced after a bleed and it plays an essential role in clotting. However, numerous studies show that thrombin causes cell death, inflammation, blood–brain barrier disruption, edema, and so on, as demonstrated by studies that directly infuse thrombin into the brain (reductionist approach) and by ICH studies that find thrombin inhibitors to be neuroprotective.[33,34] Importantly, Kawai and colleagues[35] have reported that mild hypothermia reduced edema, inflammation, and blood–brain barrier damage after thrombin infusion. Nonetheless, the present study retested the hypothesis that mild hypothermia mitigates thrombin-mediated edema, and we also assessed brain injury and functional impairment at a longer survival time. Our overarching concern is that mild hypothermia fails to markedly and consistently improve histological and behavioral outcome after ICH in rodents due to side effects [e.g., aggravating bleeding][19], inadequate protection against certain key elements of an ICH (e.g., thrombin-mediated neurotoxicity), and modeling limitations. On the latter, for example, it may be that rodent ICH studies have used insults that cause edema and ICP elevations that are of little direct importance to cell death, survival, and long-term behavioral outlook.

2.2 Methods

Fifty-six male Sprague–Dawley rats (250–300 g, ~10-week old) were obtained from a University of Alberta colony and used according to Canadian Council of Animal Care Guidelines. As well, the Biosciences Animal Care and Use committee at the University of Alberta approved all procedures. Rats were housed on a 12-hour light/dark cycle with *ad libitum* food and water in a temperature- and humidity-controlled room. Animals were randomly assigned to groups, and an investigator blinded to group identity conducted all histological and behavioral procedures.

Two experiments were done to assess the effects of systemic (Expt. 1) and local brain hypothermia (Expt. 2) on BWC after an intra-striatal thrombin infusion (n = 8 per group). These rats were euthanized at 24 hours after thrombin infusion, which usually corresponds to peak edema time in this model. The third experiment tested whether systemic hypothermia persistently influenced cell death and behavioral impairment in thrombin-infused rats that survived for 14 days (n = 12 per group).

Temperature probe implantation (Expt. 1 and 3)

Four days prior to thrombin injection the rats were anesthetized with isoflurane (4% induction, 1.5–2% maintenance; 60% N_2O and remainder O_2), an incision was made into the peritoneal cavity, and a telemetry probe was inserted to measure core temperature (TAT10TA-F20/F40; Transoma Medical).[36] The wound was sutured and treated with the analgesic Marcaine, and the rats were singly housed over receivers (RPC-1; Transoma Medical) that collected temperature data. Data from the day prior to thrombin surgery served as their baseline.

Telemetry probes were calibrated to within 0.2°C based upon a thermocouple probe and calibration-grade glass thermometer.

Thrombin surgery

Rats were anesthetized with isoflurane and temperature was maintained at 37°C with a heated water pad and rectal temperature probe. A midline scalp incision was made, and the head was balanced in a stereotaxic frame. A burr hole was drilled at 0.5 mm anterior and 3.5 mm to the right of Bregma, and a 26G Hamilton syringe was inserted into the striatum (6.5 mm below the skull surface). Over 10 minutes, 2.5 U (Expt. 1) or 1 U (Expts. 2 and 3) of rat thrombin in 30 µL of sterile saline was infused (Sigma; No. T5772). Doses were titrated by batch to produce roughly comparable injury (pilot data not shown). The needle was left in place for an additional 10 minutes to prevent fluid backflow after which a metal screw was used to seal the drill hole, and the wound was closed with wound clips and Marcaine was applied. Body temperature was monitored following surgery via telemetry in Expts. 1 and 3.

Therapeutic hypothermia

Whole-body hypothermia (Expts. 1 and 3) was produced in freely moving rats with a system that uses a heat lamp, water mister, and a fan based upon temperature data from a telemetry probe sent to a computer running ART 2.3 (Transoma Medical) and custom purpose software.[36] Cooling began one hour after thrombin infusion. Rats were cooled over the course of an hour to 33°C and kept there until euthanasia at 24 hours post-infusion (Expt. 1). Rats in Expt. 3 were cooled for 72 hours and rewarmed by 0.5°C per hour over 6 hours. In Expts. 1 and 3, the normo-thermic group had their temperature monitored to ensure it was > 36°C.

For local brain hypothermia (Expt. 2), the rats were surgically implanted with a cooling coil underneath the temporalis muscle ipsilateral to the thrombin infusion.[14,15,37] This procedure immediately followed thrombin infusion. After anesthesia, the coil was connected to a water source so that cold water flowed through the coil thereby causing hypothermia in the cortex and striatum underneath the device. Cooling began one hour after thrombin infusion, and based upon published data the rats' affected hemisphere would have been cooled within minutes to ~31–33°C and kept at this level until euthanasia.[14,15,37,38] The other hemisphere and the body were expected to remain at normal temperature, which potentially offers an advantage by avoiding systemic side effects. Note that for the normothermic group in Expt. 2 we assumed that they maintained their own body temperature based upon our experience with this model and method, including data in Expts. 1 and 3 that show the untreated rats all maintained their body temperature at normal.

The one-hour delay to cooling was used because hypothermia would be inevitably delayed in clinical practice, and thus cooling would be given after the bulk of thrombin production in a natural hemorrhage. As well, previous animal studies using models of ICH used delays of an hour or more.[2] Prolonged mild cooling was used as it has consistently provided substantial protection in our ischemia studies.[8,39,40] In the edema experiments, cooling was maintained until euthanasia thereby ensuring that we did not miss a purely symptomatic effect of cooling (i.e., that disappeared upon rewarming) or any potential rewarming complications (e.g., rebound edema). Likewise, slow rewarming was used in the third experiment to avoid such complications.

Brain water content

Rats were anesthetized (4% isoflurane) and quickly de-capitated at 24 hours after thrombin infusion. The brain was removed and a 6-mm-thick coronal section was taken from 2 mm anterior to 4 mm posterior to the injection site and dissected into cortical and striatal pieces. The cerebellum was also taken for a control. Samples were immediately weighed (wet weight), baked for 24 hours at 100°C, and then re-weighed (dry weight). The BWC was determined as follows:

[(wet weight - dry weight)/wet weight)] × 100. Increases above normal levels (~78% water) arise from edematous brain tissue.

Behavioral testing

The horizontal ladder test was used because it is sensitive to impaired walking ability caused by striatal injury.[41]Behavioural testing occurred during the light cycle and animals did four training crosses the day prior to baseline testing. To reduce stress, animals were acclimatized to the testing room for 30 minutes prior to starting behavioural procedures. Sugar pellets, as well as a shelter structure, were used to motivate crossing the ladder. At baseline and day 14, rats were videotaped crossing a 1-m ladder with metal rungs (3-mm diameter) placed 1–4 cm apart at varying distances. At each time rats were allowed to cross the ladder four times, which was video re-corded for determining the percent of successful steps. Rats that crossed fewer than three times at either time were excluded from analysis for this test.

Histology

Rats were deeply anesthetized (pentobarbital, 100 mg/kg, i.p.) and euthanized by cardiac perfusion with 0.9% saline followed by 10% formalin. Brains were extracted and subsequently cryosectioned at 40 μm and then stained with cresyl violet. One in every 10 sections was

analyzed with ImageJ starting anterior to the lesion and ending posterior to the lesion.[40,41] Lesion volume was assessed using the following formulas.

Volume of a hemisphere = average (area of the complete coronal section of the hemisphere - area of ventricle - area of damage) × interval between sections × number of sections.

Tissue lost = remaining volume of normal hemisphere - remaining volume of injured hemisphere.

Neuronal death in the peri-infarct/cavity zone was assessed by counting cells labeled with Fluoro-Jade B.[42] Sections were incubated with 0.001% Fluoro-Jade (Chemicon) and labeled cells were visualized with a fluorescence microscope. All cells were counted in one coronal section per animal at the level of maximum injury.[43]

Statistical analysis

Data are presented as mean ± standard deviation (SD) and were analyzed by analysis of variance, *t*-tests (equal variances not assumed), or nonparametric statistics (Wilcoxon signed rank test and Mann–Whitney *U* test) using SPSS (v. 21; SPSS, Inc.).

2.3 Results

Experiment one

One rat assigned to the hypothermia group died during thrombin surgery for an unknown reason. There were no other exclusions.

Baseline core temperature was normal at 37.3°C ± 0.16 (mean ± SD) and 37.2°C ± 0.12 in the normothermic (NORMO) and hypothermic (HYPO) groups, respectively, prior to thrombin surgery ($p = 0.154$). Temperature was regulated as desired after thrombin surgery (Fig. 1(A)). Thrombin caused a significant increase in BWC in the striatum and cortex ($p < 0.001$ vs. contralateral side, Fig. 2(A)). Systemic hypothermia did not significantly affect BWC in any structure ($p \geq 0.086$). The cerebellum had normal values, as expected.

Experiment two

Three animals were excluded due to either mortality or cooling device failure. The cause of mortality was unknown, but one occurred in each group.

Thrombin caused a significant increase in BWC in the striatum and cortex ($p < 0.001$ vs. contralateral side, Fig. 2(B)). Hypothermia did not significantly affect BWC in any structure ($p \geq 0.130$). The cerebellum had normal values, as expected.

Experiment three

One rat from the NORMO group died during thrombin surgery for an unknown reason. Four rats were excluded from ladder analyses because they did not make sufficient walks across the ladder (two rats from each group). Finally, one hypothermic rat was excluded due to battery failure in the telemetry probe.

Baseline core temperature was normal and averaged 37.1°C ± 0.29 and 37.1°C ± 0.30 in the NORMO and HYPO groups, respectively, prior to thrombin surgery ($p = 0.591$). Temperature was regulated as desired after thrombin surgery (Fig. 1(B)).

The ladder test failed to statistically detect impairment with the contralateral-to-infusion forelimb (baseline vs. day 14, $p = 0.075$, Fig. 3(A)), and there was no group difference at baseline ($p = 0.673$) or day 14 ($p = 0.059$). However, the hind limb was significantly impaired after thrombin infusion ($p = 0.001$, Fig. 3(B)), and there was a group main effect ($p = 0.014$). However, as there was a trend toward a baseline difference in these hind limb scores ($p = 0.066$), we analyzed the day-14 data as a percent of baseline, which then confirmed that there was no significant group difference ($p = 0.379$). Thus, hypothermia did not significantly alter the error rate on the ladder test.

Thrombin injection caused considerable tissue loss largely affecting striatum, which was unaffected by hypothermia ($p = 0.702$, Fig. 4(A), (B)). However, cooling did result in a significantly greater number of Fluoro-Jade positive cells compared with normothermic rats ($p = 0.035$, Fig. 4(C), (D)). In this control group, all but one rat had no Fluoro-Jade-positive cells whereas that one rat had just two cells in the peri-lesion zone.

2.4 Discussion

A moderate thrombin infusion into striatum caused cell death, behavioral dysfunction, and cerebral edema, as others report.[33,34] However, unlike an earlier study,[35] mild hypothermia failed to lessen edema in our experiments. Similarly, hypothermia failed to lessen behavioral impairment or reduce brain injury after thrombin infusion. These findings suggest that mild hypothermia will not effectively treat thrombin-induced secondary injury after an ICH, which may help explain the inconsistent, lackluster, or nonexistent histological protection found in rodent ICH studies.

Contrary to our hypothesis, hypothermia increased peri-hematoma neuronal death at 14 days. While it appears that hypothermia worsens cell death, we expect that cooling simply delays injury, which can occur after global ischemia.[39,44] In support of this argument are findings that thrombin does not normally cause chronic cell death,[45] which we confirmed in the normothermic thrombin-infused rats. That coupled with the fact that lesion volume and behavioral deficits were the same at 14 days argues against a worsening of injury. Nonetheless, it is possible that cooling worsens peri-lesion neuronal death, which may become evident with lesion volume measurement only at longer survival times (not presently assessed). If so, then further work is needed to identify the underlying cause.

Our study does not explain why others found hypothermia to lessen thrombin-induced edema, inflammation, and blood– brain barrier disruption[35] whereas we found no protection. While both studies are similar (comparable levels of edema, same sex and strain of rats, etc.), there are differences that might explain this discrepancy. First, Kawai and others[35] used a 10-U dose of bovine thrombin (10 U) whereas we used 1 and 2.5 U of rat thrombin. In our hands rat thrombin produces excessive brain injury and mortality at doses of 5 U and above (unpublished data). We chose rat thrombin because it should have better face validity than using bovine thrombin in rats. Second, the anesthetic was different between studies (pentobarbital vs. isoflurane), which might have influenced outcome through a direct neuroprotective effect or indirectly (e.g., pentobarbital would facilitate postoperative cooling). Third, they induced cooling by placing rats in a cold room, which they found caused brain temperature to be 30–35°C. However, this was measured in otherwise naive rats, and the thrombin insult coupled with use of pentobarbital might have resulted in a greater level of hypothermia. This may be more efficacious but it is of greater risk to patients so we only tested mild cooling. Fourth, we delayed

cooling for one hour whereas Kawai and others[35] appear to have initiated hypothermia immediately, which may be more protective. We chose a one-hour delay because it has better clinical relevance. Note, however, that our brain-selective cooling reaches target temperature within a few minutes so we did not have an overly long intervention delay. Nonetheless, owing to these and potentially other factors (e.g., inflammatory response to bovine vs. rat thrombin), we cannot isolate why our results differ from Kawai and others[35].

Our work, as with any negative study, has certain limitations to consider. Notably, a neuroprotective effect may have been found had we used another endpoint or test time, cooling dose or method [e.g., drugs[22]], a different insult severity, or larger group sizes. While we cannot exclude these possibilities, we did use reasonable group sizes and three established endpoints (behavior, edema, and two histology measures) at appropriate survival times, and we conducted three separate experiments that all yielded the same general finding (no protective effect was found). We also used two cooling methods and protocols (depth and duration) that we find provide superior protection against global and focal ischemia,[38,46] and that reduces edema after ICH.[14,20] These protocols also have good face validity to current clinical protocols (e.g., depth and duration of treatment).[11,12,24,25] Note that we used local cooling to minimize cardiovascular complications[37] that might impact outlook (e.g., altering BP). We did not, however, assess whether local cooling would reduce cell death given the negative findings against thrombin-induced edema and the rest of our data.

Another important concern centers on the use of simplified reductionist models, specifically infusing just thrombin in saline. We took this established approach[34] to isolate whether cooling directly affects thrombin-mediated injury. However, there are important differences between this simplified predictive model, more realistic isomorphic models

(collagenase and whole blood), and a natural ICH, including dose and timing of thrombin production, spatial distribution of thrombin, and the other factors present in blood but not in our infusion solution that would affect thrombin's activity (e.g., anti-thrombin III). The interaction between thrombin and other mechanisms of secondary de-generation must also be considered. Such limitations obviously must temper conclusions drawn regarding the true efficacy of hypothermia after an ICH based solely upon the thrombin model. As well, we did not include a control group of non-thrombin induced injury; however, we followed the same thrombin-infusion protocol previously that found that heat-inactivated thrombin did not cause damage beyond the needle insertion.[45]

In summary, we report that mild and prolonged hypothermia does not attenuate thrombin-induced edema, cell death, or behavioral dysfunction. Given the aforementioned limitations, we do not advise using just the thrombin model to predict the clinical efficacy of hypothermia for an ICH. For that we rely upon the numerous studies that use animal models of ICH as these have better face validity. Instead, the present findings help provide a mechanistic understanding of the strengths and weaknesses of using therapeutic hypothermia for ICH. Identifying weaknesses could lead to more effective therapies, such as using cotreatments to target those mechanisms of injury not attenuated by cooling. Finally, these data help explain why cooling does not provide as much benefit against ICH as one might expect based upon the ability of hypothermia to mitigate injury after ischemia that shares several mechanism of injury.[29,32-34] Likely this is because our cooling protocols are not effective against two key mechanisms of secondary degeneration after ICH – thrombin production as presently observed and iron toxicity (unpublished data). This raises the question of exactly how cooling reduces edema, inflammation, and blood– brain barrier injury after ICH.

2.5 References

1. van der Worp, H. Bart, Sena ES, Donnan GA, Howells DW, Macleod MR. Hypothermia in animal models of acute ischaemic stroke: A systematic review and meta-analysis. Brain 2007 Dec;130(Pt 12):3063-3074.

2. MacLellan CL, Peeling J, Colbourne F. Cytoprotection strategies for experimental intracerebral hemorrhage (ch 19).
. In: Carpuahoma JR, Mayer SA, Hanley DF, editors. Textbook of intracerebral hemorrhage. New York: Cambridge University Press; 2009.

3. van der Worp, H. Bart, Macleod MR, Kollmar R. Therapeutic hypothermia for acute ischemic stroke: Ready to start large randomized trials? J Cereb Blood Flow Metab 2010 Jun;30(6):1079-1093.

4. Stroke Therapy Academic Industry Roundtable. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 1999 Dec;30(12):2752-2758.

5. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp, Bart H, Howells DW. 1,026 experimental treatments in acute stroke. Annals of Neurology 2006 Mar;59(3):467-477.

6. Macleod MR, Fisher M, O'Collins V, Sena ES, Dirnagl U, Bath PMW, et al. Good laboratory practice: Preventing introduction of bias at the bench. Stroke 2009 Mar;40(3):50.

7. Lapchak PA, Zhang JH. Resolving the negative data publication dilemma in translational stroke research. Transl Stroke Res 2011 Mar;2(1):1-6.

8. MacLellan CL, Clark DL, Silasi G, Colbourne F. Use of prolonged hypothermia to treat ischemic and hemorrhagic stroke. J Neurotrauma 2009 Mar;26(3):313-323.

9. The Hypothermia after Cardiac Arrest Study Group. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. The New England Journal of Medicine 2002 Feb 21,;346(8):549-556.

10. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med 2005 Oct 13,;353(15):1574-1584.

11. Lyden PD, Hemmen TM, Grotta J, Rapp K, Raman R. Endovascular therapeutic hypothermia for acute ischemic stroke: ICTuS 2/3 protocol. International Journal of Stroke 2014 Jan;9(1):117-125.

12. van der Worp, H. Bart, Macleod MR, Bath PMW, Demotes J, Durand-Zaleski I, Gebhardt B, et al. EuroHYP-1: European multicenter, randomized, phase III clinical trial of therapeutic hypothermia plus best medical treatment vs. best medical treatment alone for acute ischemic stroke. International Journal of Stroke 2014 Jul;9(5):642-645.

13. Dai D, Wang D, Li K, Mao Y, Zhang L, Duan S, et al. [Effect of local mild hypothermia on expression of aquaporin-4 following intracerebral hemorrhage in rats]. Zhonghua Yi Xue Za Zhi 2006 Apr;86(13):906-910.

14. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

15. Fingas M, Penner M, Silasi G, Colbourne F. Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia. Experimental Neurology 2009;219(1):156-162.

16. Kawanishi M. Effect of hypothermia on brain edema formation following intracerebral hemorrhage in rats. Acta Neurochir Suppl 2003;86:453-456.

17. Kawanishi M, MD, Kawai N, MD, Nakamura T, MD, Luo C, MD, Tamiya T, MD, Nagao S, MD. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. Journal of Stroke and Cerebrovascular Diseases 2008;17(4):187-195.

18. MacLellan C, Shuaib A, Colbourne F. Failure of delayed and prolonged hypothermia to favorably affect hemorrhagic stroke in rats. Brain Research 2002;958(1):192-200.

19. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

20. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

21. Wagner KR, Beiler S, Beiler C, Kirkman J, Casey K, Robinson T, et al. Delayed profound local brain hypothermia markedly reduces interleukin-1beta gene expression and vasogenic edema development in a porcine model of intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:177-182.

22. Wei S, Sun J, Li J, Wang L, Hall CL, Dix TA, et al. Acute and delayed protective effects of pharmacologically induced hypothermia in an intracerebral hemorrhage stroke model of mice. Neuroscience 2013;252:489-500.

23. Feng H, Shi D, Wang D, Xin X, Feng L, Zhang Y, et al. [Effect of local mild hypothermia on treatment of acute intracerebral hemorrhage, a clinical study]. Zhonghua Yi Xue Za Zhi 2002 Dec 10,;82(23):1622-1624.

24. Kollmar R, Staykov D, Dörfler A, Schellinger PD, Schwab S, Bardutzky J. Hypothermia reduces perihemorrhagic edema after intracerebral hemorrhage. Stroke 2010 Aug;41(8):1684-1689.

25. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

26. Rincon F, Friedman DP, Bell R, Mayer SA, Bray PF. Targeted temperature management after intracerebral hemorrhage (TTM-ICH): Methodology of a prospective randomized clinical trial. Int J Stroke 2014 Jul;9(5):646-651.

27. Janny P, Papo I, Chazal J, Colnet G, Barretto LC. Intracranial hypertension and prognosis of spontaneous intracerebral haematomas. A correlative study of 60 patients. Acta Neurochir (Wien) 1982;61(1-3):181-186.

28. Venkatasubramanian C, Mlynash M, Finley-Caulfield A, Eyngorn I, Kalimuthu R, Snider RW, et al. Natural history of perihematomal edema after intracerebral hemorrhage measured by serial magnetic resonance imaging. Stroke 2011 Jan;42(1):73-80.

29. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

30. Arima H, Wang JG, Huang Y, Heeley E, Skulina C, Parsons MW, et al. Significance of perihematomal edema in acute intracerebral hemorrhage: The INTERACT trial. Neurology 2009 Dec;73(23):1963-1968.

31. Hiploylee C, Colbourne F. Intracranial pressure measured in freely moving rats for days after intracerebral hemorrhage. Exp Neurol 2014 May;255:49-55.

32. Kirkman MA, Allan SM, Parry-Jones AR. Experimental intracerebral hemorrhage: Avoiding pitfalls in translational research. J Cereb Blood Flow Metab 2011 Nov;31(11):2135-2151.

33. Babu R, Bagley JH, Di C, Friedman AH, Adamson C. Thrombin and hemin as central factors in the mechanisms of intracerebral hemorrhage-induced secondary brain injury and as potential targets for intervention. Neurosurg Focus 2012 Apr;32(4):E8.

34. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

35. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

36. DeBow S, Colbourne F. Brain temperature measurement and regulation in awake and freely moving rodents. Methods 2003 Jun;30(2):167-171.

37. Clark DL, Colbourne F. A simple method to induce focal brain hypothermia in rats. J Cereb Blood Flow Metab 2007 Jan;27(1):115-122.

38. Clark DL, Penner M, Wowk S, Orellana-Jordan I, Colbourne F. Treatments (12 and 48 h) with systemic and brain-selective hypothermia techniques after permanent focal cerebral ischemia in rat. Exp Neurol 2009 Dec;220(2):391-399.

39. Colbourne F, Corbett D. Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil. Brain Res 1994 Aug 22,;654(2):265-272.

40. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

41. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

42. Schmued LC, Hopkins KJ. Fluoro-jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 2000 Aug;874(2):123-130.

43. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

44. Dietrich WD, Busto R, Alonso O, Globus MY, Ginsberg MD. Intraischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. J Cereb Blood Flow Metab 1993 Jul;13(4):541-549.

45. Caliaperumal J, Brodie S, Ma Y, Colbourne F. Thrombin causes neuronal atrophy and acute but not chronic cell death. Can J Neurol Sci 2014 Nov;41(6):714-720.

46. Silasi G, Colbourne F. Unilateral brain hypothermia as a method to examine efficacy and mechanisms of neuroprotection against global ischemia. Ther Hypothermia Temp Manag 2011;1(2):87-94.

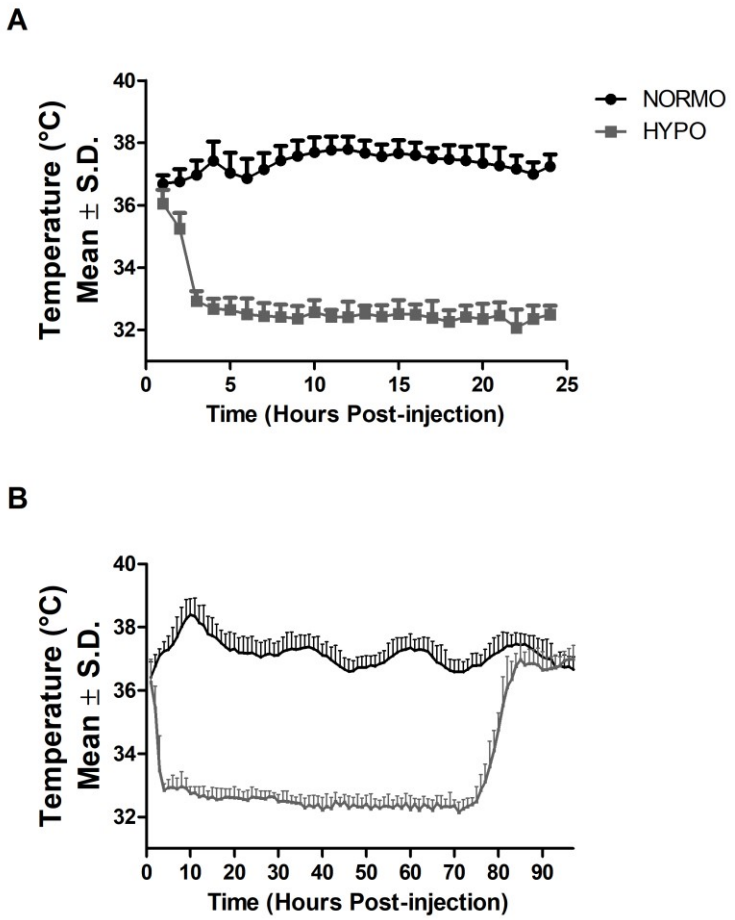

Fig. 1. Core temperature (°C, mean ± standard deviation) was measured with an implanted telemetry probe and post-thrombin data for the normothermic (NORMO) and hypothermic (HYPO) groups are shown for the (A) first (n = 7–8 per group) and (B) third experiments (n = 11 per group). Baseline temperature prior to this surgery was normal (average of ~37.2°C, not shown). In Expt. 1, systemic hypothermia was induced starting one hour after infusion and kept at ~32.5°C until euthanasia at 24 hours. In Expt. 3, systemic cooling lasted for 72 hours and these rats were euthanized on day 14. Fluctuations in temperature over time in normothermic rats are largely circadian in nature. Animals in the second experiment were subjected to focal brain cooling and did not have body temperature recorded.

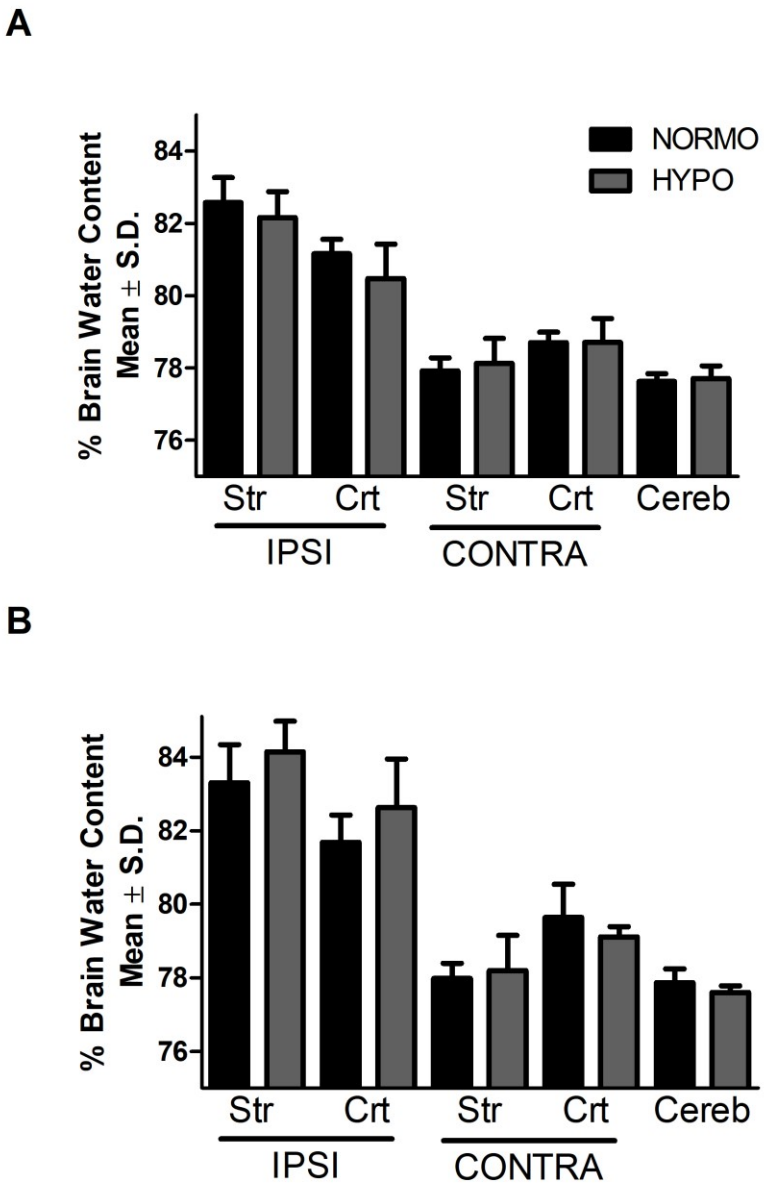

Fig. 2. Thrombin caused significant increases in striatal (Str) and cortical (Crt) brain water content (BWC) in the (A) first (n = 7-8 per group) and (B) second experiments (n = 6-7 per group) at a 24-hour survival ($p < 0.001$ vs. contralateral undamaged side). The contralateral regions and the cerebellum (Cereb) were normal. (A) Systemic and (B) local brain hypothermia did not significantly affect BWC ($p \geq 0.086$).

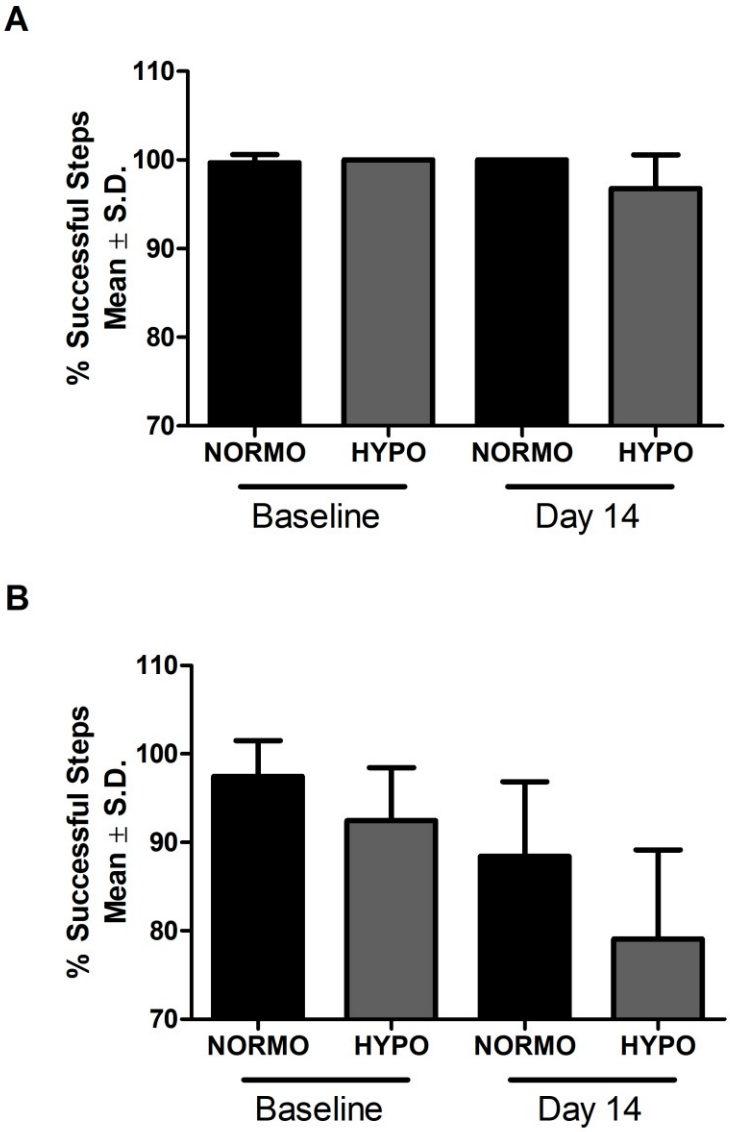

Fig. 3. Thrombin-induced brain injury increased stepping error rate on the ladder test with the contralateral (A) fore and (B) hind limbs, but this was only statistically significant for the hind limb ($p = 0.001$ for day 14 vs. baseline, n = 8-9 per group). Hypothermia appeared to significantly worsen this error rate on day 14, but this was not statistically significant when error rate was analyzed as a percent of base-line scores (NORMO: 90.8 ± 7.9, HYPO: 86.0 ± 12.8; $p = 0.379$). Thus, cooling did not affect error rate with either limbs.

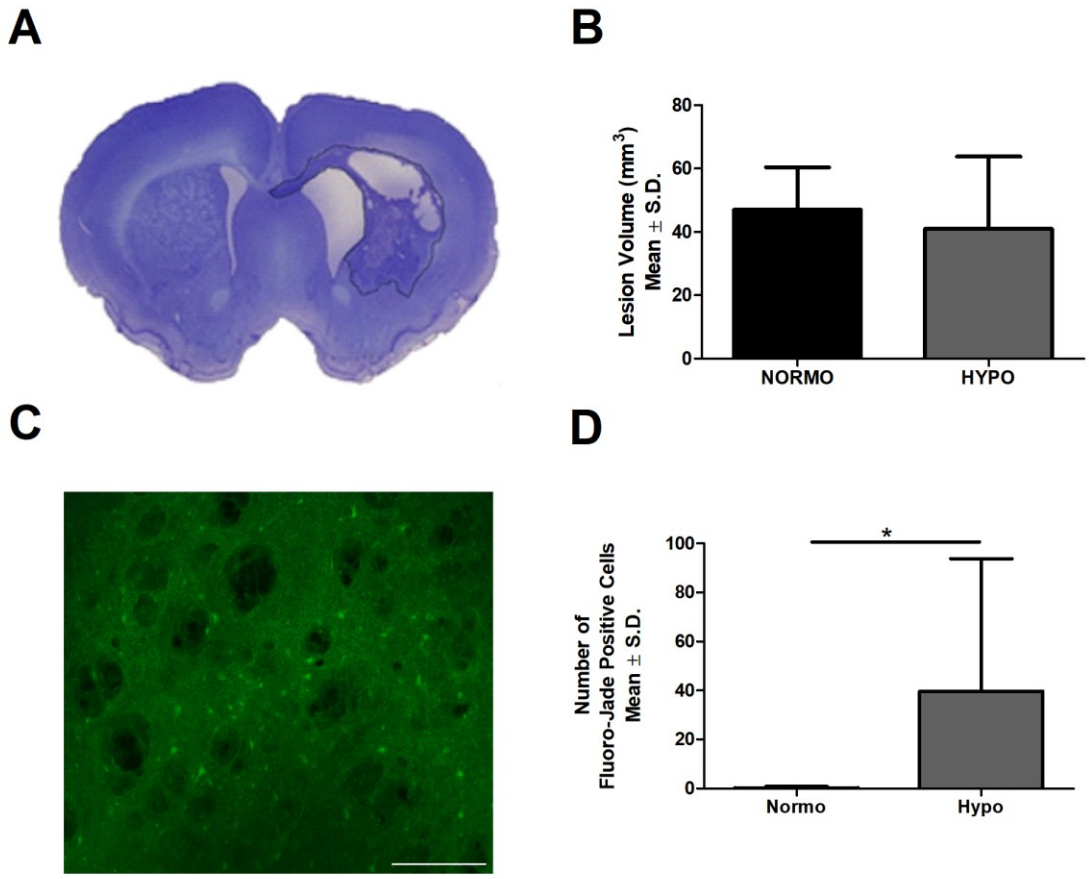

Fig. 4. (A) The photomicrograph illustrates brain injury 14 days after thrombin infusion (coronal section at level of maximal injury shown stained with cresyl violet). A *black line* demarcates the lesion zone, which was quantified with ImageJ. At this survival time, thrombin resulted in an obvious striatal lesion with ventriculomegaly. (B) Systemic hypothermia did not affect lesion volume ($p = 0.702$, $n = 11$ each). (C) A photomicrograph illustrating Fluoro-Jade B positive cells (de-generating neurons) in the peri-lesion zone of a hypothermia-treated animal (scale bar = 50 μm). Only one of the normothermic rats had a small number of Fluoro-Jade B-positive cells; the rest had none. (D) This peri-lesion neuronal death was significantly greater at 14 days in hypothermic rats (*$p = 0.035$).

Chapter Three

Therapeutic Hypothermia Does Not Mitigate Iron-Induced Injury in Rat

3.1 Introduction

Therapeutic hypothermia (TH) mitigates injury and improves survival from cardiac arrest and neonatal hypoxic ischemic encephalopathy. Therefore, there is considerable interest for using it after traumatic brain and spinal cord injury, ischemic stroke, and hemorrhagic stroke.[1] This interest has grown from extensive positive findings in animal models testing efficacy and mechanisms of action.[2] From this it seems that TH abates numerous mechanisms of injury, although unequally, so much so that it should be considered a complex cocktail therapy. Indeed, cooling may be ineffective against some mechanisms while potentially aggravating others. Thus, it makes sense to identify those strengths and weaknesses in an effort to further optimize outcome through cotreatment.[3] Clinical interest for large intracerebral hemorrhage (ICH) is based upon data showing that cooling lessens edema and raised ICP after ischemic stroke.[2] Furthermore, small clinical studies support the use of TH after ICH,[4-6] as do animal studies, which find TH to reduce edema[7-11] and ICP[12] along with other protective effects.[9,10,13,14] Nonetheless, TH may not effectively target all mechanisms of injury, which probably varies with treatment parameters, models, etc. For instance, some find that TH reduces the harmful effects of thrombin,[15] whereas others do not.[16]

It is widely accepted that iron toxicity substantially contributes to secondary damage after ICH.[17] Free iron, originating from the breakdown of hemoglobin, catalyzes the production of hydroxyl radicals (oxidative stress). Iron levels increase and remain persistently elevated in the brain after ICH, and this causes oxidative injury.[17] Further evidence from animal studies show that free radical scavengers and iron chelators, such as deferoxamine,[18] are neuroprotective. Moreover, direct infusion of iron, such as $FeCl_2$, is neurotoxic, thereby adequately modeling iron toxicity following ICH. Notably, $FeCl_2$ infusions cause oxidative stress, edema, and acute and

chronic cell death as seen in ICH models.[19-21] We hypothesized that cooling would directly attenuate free radical production (through slowing chemical production) while also mitigating downstream effects (e.g., edema) that contribute to injury. Thus, we used a simple model, similar to our recent thrombin study,[16] to assess TH against iron ($FeCl_2$)-mediated toxicity. Use of simple models, such as this, is common in stroke research, including for ICH (e.g., balloon inflation model and the infusion of individual blood components), where the goal is to isolate a particular mechanism for study.

3.2 Methods

Animals and experimental conditions

All procedures were conducted in accordance with the Canadian Council on Animal Care under the approval of the University of Alberta's Biosciences Animal Care and Use Committee. One hundred fourteen male Sprague Dawley rats (250-350 g, ~10-11 weeks old) were housed in cages with wood chip bedding in a temperature and humidity controlled room on a 12-hour light cycle. Animals were given *ad lib* water and food (Rodent Chow, Lab Diet), except during certain testing conditions. Animals were randomized into normothermia (NORMO) or hypothermia (HYPO) groups, and most data were analyzed blinded to group identity. However, blinding was sometimes impossible (e.g., euthanizing rats when they were still cold). Several experiments were done. First, we evaluated whether TH affected bleeding (n = 8/group). Second, we assessed whether TH affected edema at 24 (n = 11/group) and 72 hours postinjury (n = 8/group). Lastly, we determined whether TH impacted tissue loss and behavioral impairments at 7 (n = 14/group, experiment 3) and 28 days postinjury (n = 16/group, experiment 4).

Temperature probe surgery

All rats had a temperature probe (TA10TA-F20 or F40 Transoma Medical) implanted into their peritoneal cavity 4–5 days before $FeCl_2$ infusion. Briefly, rats were anesthetized with isoflurane (4% induction and 1.5-2% maintenance in 60% N_2O and balance O_2) and a small midline abdominal incision was made for insertion of the sterilized transmitter. The wound was sutured and Marcaine was used as a local anesthetic. Rats were placed in separate cages over receivers (RPC-1; Transoma Medical) for data collection.[10] Temperature was sampled every 30 seconds through ART 2.3 (Transoma Medical). Probes were calibrated to within 0.2°C accuracy. All rats tolerated this procedure well, and there were no signs of surgical complications.

Iron infusion surgery

Rats were anesthetized with isoflurane and a midline scalp incision was made. Using a stereotaxic frame, a hole was drilled 0.5 mm anterior and 3.5 mm lateral to Bregma. A Hamilton syringe was lowered 6.5 mm from the skull surface and 3.8 µg of $FeCl_2$ in a 30 µL solution of sterile unbuffered saline was infused over 10 minutes, during which time the body temperature was regulated at normothermia by a heating pad.[19,20] The needle was left in place for an additional 10 minutes to prevent backflow. The scalp was sutured and Marcaine was applied. Rats were then placed in their cage for temperature monitoring.

It should be noted that the insertion of a needle into the brain unavoidably causes a small hemorrhage (a few micro-liters) and minor brain injury. The slow infusion of 30 µL of saline or acidic saline (to match the $FeCl_2$ infusion), however, appears to be of little additional consequence.[19,22] Thus, a $FeCl_2$ infusion causes brain injury and related events almost entirely from iron-mediated oxidative stress.

Temperature monitoring and control

Baseline temperature was recorded on the day before $FeCl_2$ infusion surgery. The NORMO group was only monitored after $FeCl_2$ surgery, whereas HYPO rats also had their temperature regulated by a servo-regulated system consisting of a fan, water mister, and an infrared heat lamp.[23] These devices were situated above the rat's cage, and whole-body hypothermia was precisely maintained in freely moving animals (no anesthesia). The HYPO rats were cooled to ~33.0°C over 1 hour beginning 1 hour after iron infusion. They were kept at this level until euthanasia at 24 or 72 hours at which time edema was measured (experiments 1 and 2). In experiments 3 and 4, rats were cooled for 72 hours then rewarmed over 6 hours (0.5°C/hour). In our laboratory, such protocols give superior protection in models of global[24] and focal ischemia,[25] and they appear to have no lasting behavioral or histological effects in normal animals.[26] Although initiating TH an hour after iron infusion may not appear clinically relevant, it should be kept in mind that iron toxicity does not occur immediately after an ICH, and so cooling could easily be applied during much of the acute toxic effects. In addition, $FeCl_2$ infusion causes protracted cell death, edema, and so on, therefore, cooling was applied during this period.

Bleeding

Blood volume was evaluated with a spectrophotometric hemoglobin assay as recently done.[27] Rats were anesthetized with isoflurane and decapitated at 48 hours postinjury. Each whole hemisphere was separately homogenized in double distilled water (1:4 weight:volume ratio), left to incubate on ice, and then centrifuged at 15,800 *g* for 35 minutes. The supernatant was divided into aliquots that were reacted with Drabkin's solution (Ricca Chemical

RI266016). The absorption values (measured at 540 nm; Model 4001/4; Thermo Fisher Scientific) were compared to a standard curve based on known quantities of blood. This method measured blood present within the vasculature as well as that caused by the experimental procedures (needle insertion and infusion of iron). The contralateral-to-infusion hemisphere contained only blood within the vasculature (i.e., serves as an internal control).

Brain Water Content (i.e., Edema)

Rats were anesthetized and decapitated to determine brain water content (BWC) at 24- or 72-hour survival times. The wet weight of the ipsilateral (IPSI) and contralateral (CONTRA) striatum (from 2 mm anterior to 4 mm posterior to the injection site) and the cerebellum were taken. The brains were then baked for 24 hours at 100°C to determine dry weight. The BWC was calculated as:

$$[(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100$$

Levels > 78% is indicative of edema.

Behavioral testing

Rats in the last two experiments underwent behavioral assessment before and after surgery. All behavioural assessments occurred during the light cycle and animals were acclimatized to the room 30 minutes prior to beginning behavioural testing. In experiment 3, the corner turn test (CTT), neurological deficit scale (NDS), and the horizontal ladder test were used to evaluate deficits 7 days postinjury. In experiment 4, the horizontal ladder test was used to assess impairments 28 days postinjury, while the Montoya staircase was used on days 24-28. These tests are sensitive to striatal injury.[19,28]

The CTT consists of two walls (41 cm high by 30.5 cm wide) placed at a 30° angle with a 0.5 cm gap. At baseline, the direction each rat turned coming out of the corner was averaged over 2 days (10 trials/day). Rats with a strong turning bias (<30% in one direction) were excluded from analysis of this test data to avoid floor or ceiling effects that might mask the influence of treatment. The turning preference was determined over 10 trials during the post-insult testing session.

The NDS is a composite of several simple motor tasks (hindlimb retraction, contralateral forelimb flexion, bilateral forepaw grasp, beam walking, and spontaneous circling), each of which range in a score from 0 to 3, except the contralateral forelimb flexion that ranges from 0 to 2. Fourteen is the maximum impairment.

The ladder test measured the walking ability across a horizontal ladder (1 m) with metal rings (3 mm diameter) randomly spaced 1-4 cm apart. Rats were trained to make four traverses the day prior to baseline testing and food and shelter structure were used to motivate crossing. During baseline and day 7 or 28, they were video recorded for later determination of the number of successful steps and errors. Rats were excluded if they did not cross at least twice, and this was to ensure we had a sufficient sample to accurately estimate their performance.

The staircase test measures skilled reaching. Rats were placed in a plexiglass box (length: 30 cm; width: 6.8 cm; height: 12 cm) and trained to reach for sugar pellets (45 mg; Bio-Serv) from a bilaterally placed staircase. The number of pellets consumed with each forelimb was recorded (maxi-mum 21 pellets/side/trial) during training (two 15-min trials/day, 5 days/week, 4 weeks) and testing (24-28 days post-$FeCl_2$). The last week of training served as a baseline measurement (stable performance), and any rat that failed to reach at least eight pellets with a

paw was excluded from this test. This was done to avoid floor effects (i.e., exclude poor performers). During training and testing in the staircase test, the rats were food deprived to ~90% of their free-feeding body weight accounting for natural growth over time to encourage reaching. Body weight was repeatedly measured during this period and found to be similar between groups. In this study, food deprivation ended several days before surgery to allow rats time to regain lost weight. In addition, these rats were not food deprived again until ~3 weeks after $FeCl_2$ surgery.

Histology

After behavioral assessment at 7 or 28 days, the rats were anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused through the heart with 0.9% saline followed by 10% formalin. The brains were removed and later cryosectioned for assessment of lesion volume. For this, a 40-μm thick section was taken every 200 μm starting anterior to the lesion and ending posterior to injury. Sections were stained with Cresyl Violet and 1 in every 10 sections was analyzed using ImageJ software (Scion Corporation). The following formulas were used to determine tissue loss:[19,28]

Hemisphere volume = average (area of the complete coronal section of the hemisphere - area of ventricle - area of damage) × interval between sections × number of sections.

Lesion volume = remaining volume of normal hemisphere - remaining volume of injured hemisphere.

Statistical analyses

Descriptive statistics are given as mean ± standard deviation (SD) or medians. Data were analyzed using analysis of variance (SPSS v.21; SPSS, Inc.), except the NDS scores, which were analyzed with Mann–Whitney *U* and Wilcoxon tests.

3.3 Results

Experiment 1

No exclusions or mortality occurred in experiment 1. Infusion of $FeCl_2$ caused a small ~6 μL increase in blood volume in NORMO rats at 48 hours ($p < 0.0011$ vs. contra-lateral hemisphere), which TH attenuated (Fig. 1, $p = 0.025$). The contralateral hemispheres were similar between groups ($p = 0.659$). Temperature was controlled as desired and discussed below for Experiment 4 (representative data).

Experiment 2

One NORMO and one HYPO rat died during surgery of unknown cause. Infusion of $FeCl_2$ caused increases in striatal BWC at 24 (Fig. 2(A)) and 72 hours (Fig. 2(B)). Cooling had no effect ($p \geq 0.139$). Cerebellum control samples were normal ($p \geq 0.650$).

Experiment 3

Two HYPO-assigned rats died from surgical errors (before HYPO treatment) and one was euthanized early because its temperature did not remain normothermic after rewarming. For the CTT, one animal was excluded from each group due to baseline turning bias. At 7 days postinjury, no behavioral deficits were detected with the CTT (Fig. 3(A)) because there was no Time ($p = 0.214$), Group ($p = 0.247$), or interaction ($p = 0.548$) effects. For the NDS, there was a significant post-infusion impairment (Fig. 3(B), $p = 0.001$), but no treatment effect on day 7 ($p =$

0.120). For the forelimb success rate on the ladder test (Fig. 3(C)), there were significant Time ($p < 0.001$) and Group effects ($p = 0.047$), but no interaction ($p = 0.978$). As baseline forelimb scores differed ($p = 0.047$), we analyzed the day 7 data as a percent of baseline, which were not different between groups ($p = 0.787$). Thus, cooling had no impact on forelimb walking errors. The success rate for the hind limb was significantly different over time (Fig. 3(D), $p < 0.001$), and there were significant Group ($p = 0.010$) and interaction effects ($p = 0.008$). While there was no difference in baseline scores ($p = 0.963$), there was a significant difference at day 7 ($p = 0.004$) when HYPO rats had greater walking impairment. Finally, $FeCl_2$ caused substantial striatal damage by 7 days (Fig. 4(A), (B)), which TH did not significantly mitigate ($p = 0.110$, Fig. 4(C)).

Experiment 4

No mortality occurred in this experiment. Figure 5 contains the post-infusion temperature data that are also representative of the other experiments, but note that HYPO rats were euthanized before rewarming in experiments 1 and 2. Baseline temperature data were normal in all cases (not shown).

There were significant walking impairments on the ladder test, but no effect of TH. This was evident by the significant Time effects ($p < 0.001$) with the nonsignificant Group ($p \geq 0.688$) and interaction effects ($p \geq 0.193$) for the affected forelimb (Fig. 6(A)) and hind limb (Fig. 6(B)). In total, six animals were excluded on the staircase test for failing to meet baseline criteria – 2 NORMO and 4 HYPO rats. There was a significant Time effect (Fig. 6(C), $p < 0.001$), but no Group ($p = 0.444$) or interaction effect ($p = 0.873$). Thus, rats had reaching impairments after

$FeCl_2$ infusion, but there was no effect of TH. Significant tissue loss was observed at 28 days (Fig. 7(A), (B)) and there was no effect of TH (Fig. 7(C), $p = 0.132$).

3.4 Discussion

We used the simple $FeCl_2$ model as a way to estimate the specific therapeutic effects of TH against iron-mediated secondary damage after ICH. While iron infusions caused cell death, edema, and behavioral deficits, the use of TH was ineffective. Accordingly, it follows that the beneficial effects of using TH after ICH, such as reducing edema and raised ICP, are likely not mediated through attenuating iron toxicity. This therapeutic weakness against a key mechanism of secondary degeneration might explain hypothermia's modest efficacy against ICH. As such, it may be possible to augment the benefits of TH by adding a drug that targets iron toxicity.[3]

Although this simplified model of iron-induced injury has been repeatedly used in ICH research,[19,20] it certainly has limitations, as do all such models (e.g., balloon inflation). First, these models exclude many interacting effects with other mechanisms of damage, some of which are likely sensitive to TH. Furthermore, extrapolating from the $FeCl_2$ model to ICH iscomplicated by the fact that we do not yet fully know the real impact of iron toxicity after ICH. This means, for example, that the lack of benefit against iron toxicity may not mean that much in those ICHs where iron toxicity is minimal. Second, there are specific concerns with the bolus infusion of $FeCl_2$, such as the fact that iron release from hemoglobin occurs much more slowly. Nonetheless, the $FeCl_2$ model does induce several mediators of injury that are shared with ICH, such as oxidative injury, edema, and seizures,[20] and the acute and chronic tissue loss seems similar.[19] Third, we timed TH to overlap with much of the injury caused by $FeCl_2$ and the time course over which drugs have been found effective, including chelators such as deferoxamine.[18] As well, our TH protocol roughly matches effective protocols used for ischemia[24,25] and ICH,[9,29]

including in some clinical studies.[5] Nonetheless, other TH regimens or methods may have provided more benefit or they may have worked against milder or more severe $FeCl_2$ insults. Such general possibilities exist with any negative study, and by the same logic, these concerns hold for follow-up studies to an initially positive finding. As well, $FeCl_2$ solution does have a pH (~4 pH) lower than biological brain levels; we did not include a control group that received a saline infusion of a lower pH as a previous study found that control animals receiving a saline solution with a pH of 4 or 5.2 did not have tissue damage beyond the damage of the needle insertion.[19]

The effectiveness of TH varies considerably among ICH studies owing to numerous study differences (e.g., models and protocols). Regardless, even in the best of cases, it seems that TH is considerably less effective in ICH than against ischemia. Perhaps this is because TH cannot directly impact primary (mechanical) damage and it apparently fails to mitigate several mechanisms of secondary damage, including iron (present data) and thrombin toxicity.[16] Additionally, TH may cause several side effects that limit efficacy, including aggravating bleeding.[27,29] While we excluded that possibility here (bleeding was slightly less), it is conceivable that TH blunts the upregulation of endogenous protective mechanisms that counter iron toxicity, such as ferritin expression.[17] Likewise, the anti-inflammatory effects of TH after an ICH[8,10] might not be helpful against iron toxicity, where such cells play an essential role (e.g., hematoma clearance after ICH). Such counterproductive effects may certainly occur in these models, which we are currently evaluating in ICH (e.g., whether TH affects iron release and distribution).

Although TH appears to treat high levels of edema and ICP after ICH,[4,5,12] there are significant limitations. The current findings illustrate one of these, which is the lack of benefit

against iron toxicity – a potentially key contributor to secondary damage after ICH.[17] These findings shed light on the inconsistent and limited protective properties of TH in the setting of ICH. Furthermore, they suggest a way forward, which is the combination of rationally selected treatments with TH[3] along with the careful control of physiological complications.

3.5 References

1. Marion D, Bullock MR. Current and future role of therapeutic hypothermia. Journal of neurotrauma 2009 Mar;26(3):455-467.

2. Yenari MA, Han HS. Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat Rev Neurosci 2012 Feb 22,;13(4):267-278.

3. Goossens J, Hachimi-Idrissi S. Combination of therapeutic hypothermia and other neuroprotective strategies after an ischemic cerebral insult. Curr Neuropharmacol 2014 Sep;12(5):399-412.

4. Feng H, Shi D, Wang D, Xin X, Feng L, Zhang Y, et al. [Effect of local mild hypothermia on treatment of acute intracerebral hemorrhage, a clinical study]. Zhonghua Yi Xue Za Zhi 2002 Dec 10,;82(23):1622-1624.

5. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

6. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

7. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

8. Kawanishi M. Effect of hypothermia on brain edema formation following intracerebral hemorrhage in rats. Acta Neurochir Suppl 2003;86:453-456.

9. Kawanishi M, MD, Kawai N, MD, Nakamura T, MD, Luo C, MD, Tamiya T, MD, Nagao S, MD. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. Journal of Stroke and Cerebrovascular Diseases 2008;17(4):187-195.

10. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

11. Wagner KR, Zuccarello M. Local brain hypothermia for neuroprotection in stroke treatment and aneurysm repair. Neurol Res 2005 Apr;27(3):238-245.

12. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

13. Sun H, Tang Y, Guan X, Li L, Wang D. Effects of selective hypothermia on blood-brain barrier integrity and tight junction protein expression levels after intracerebral hemorrhage in rats. Biol Chem 2013 Oct;394(10):1317-1324.

14. Sun H, Tang Y, Li L, Guan X, Wang D. Effects of local hypothermia on neuronal cell apoptosis after intracerebral hemorrhage in rats. J Nutr Health Aging 2015 Mar;19(3):291-298.

15. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

16. Wowk S, Ma Y, Colbourne F. Mild therapeutic hypothermia does not reduce thrombin-induced brain injury. Ther Hypothermia Temp Manag 2014 Dec;4(4):180-187.

17. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: Secondary brain injury. Stroke 2011 Jun;42(6):1781-1786.

18. Cui H, He H, Yang A, Zhou H, Wang C, Luo J, et al. Efficacy of deferoxamine in animal models of intracerebral hemorrhage: A systematic review and stratified meta-analysis. PLoS One 2015 -5-22;10(5).

19. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

20. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

21. Willmore LJ, Ueda Y. Posttraumatic epilepsy: Hemorrhage, free radicals and the molecular regulation of glutamate. Neurochem Res 2009 Apr;34(4):688-697.

22. Willmore LJ, Ballinger WE, Boggs W, Sypert GW, Rubin JJ. Dendritic alterations in rat isocortex within an iron-induced chronic epileptic focus. Neurosurgery 1980 Aug;7(2):142-146.

23. Colbourne F, Sutherland GR, Auer RN. An automated system for regulating brain temperature in awake and freely moving rodents. J Neurosci Methods 1996 Aug;67(2):185-190.

24. Colbourne F, Li H, Buchan AM. Indefatigable CA1 sector neuroprotection with mild hypothermia induced 6 hours after severe forebrain ischemia in rats. J Cereb Blood Flow Metab 1999 Jul;19(7):742-749.

25. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

26. Auriat AM, Klahr AC, Silasi G, MacLellan CL, Penner M, Clark DL, et al. Prolonged hypothermia in rat: A safety study using brain-selective and systemic treatments. Therapeutic Hypothermia and Temperature Management 2012 Mar 1,;2(1):37-43.

27. John RF, Williamson MR, Dietrich K, Colbourne F. Localized hypothermia aggravates bleeding in the collagenase model of intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2015 Mar 1,;5(1):19-25.

28. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

29. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

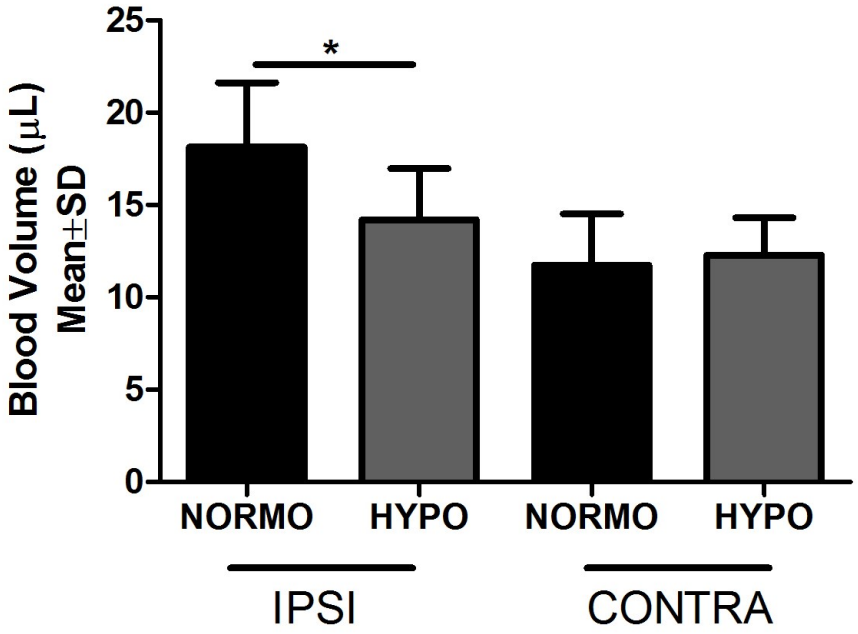

Fig. 1. The effect of therapeutic hypothermia (TH) on trauma (needle insertion) and iron-induced bleeding is shown. There was a small bleed in the NORMO group's ipsilateral (to infusion, IPSI) hemisphere. The total volume of blood in this hemisphere was attenuated by TH (*$p < 0.025$). The contralateral (CONTRA) hemisphere data show the normal amount of blood found within the cerebral vasculature (i.e., it is a control sample), which was not affected by cooling (of the IPSI hemisphere). HYPO, hypothermia; IPSI, ipsilateral; NORMO, normothermia; SD, standard deviation. n = 8/group.

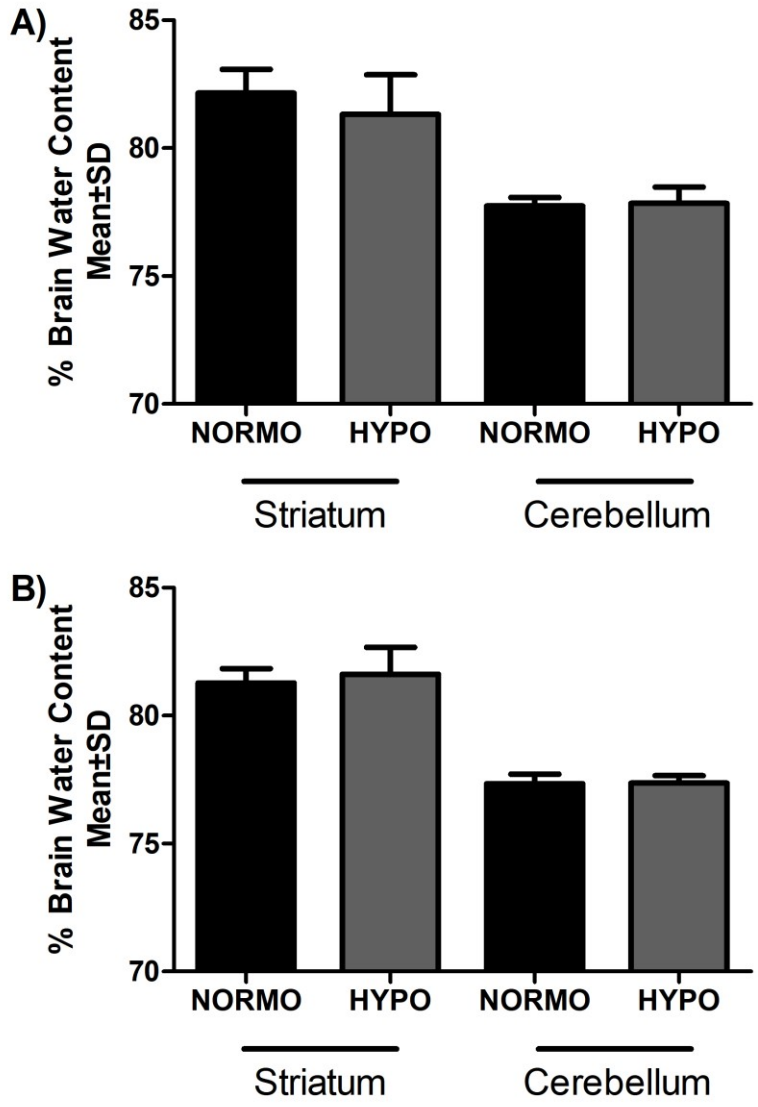

Fig. 2. Infusing $FeCl_2$ caused significant edema (i.e., above normal brain water content) at (A) 24 (n = 10/group) and (B) 72 (n = 8/group) hours, but this was not affected by TH ($p \geq 0.139$). The cerebellum control samples were unaffected, as expected.

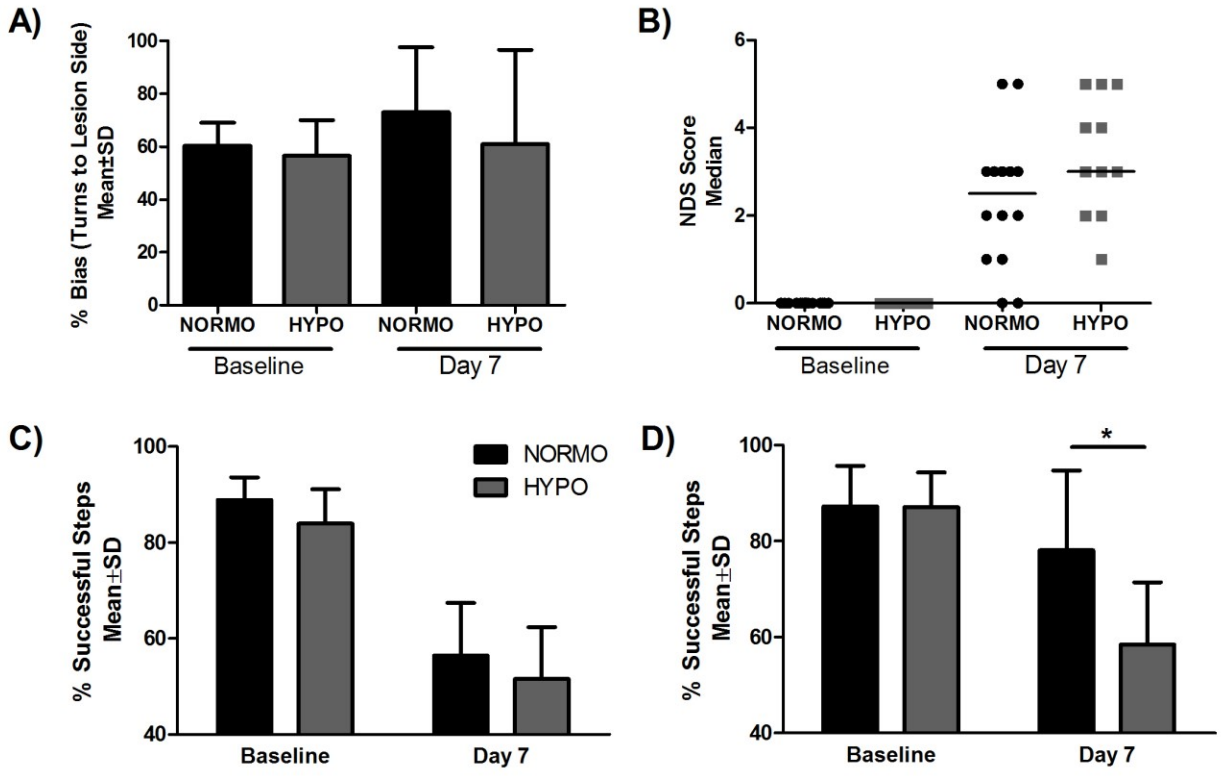

Fig. 3. The effects of TH on iron-induced behavioral deficits 7 days postinjury on the CTT (A), NDS (B), and the ladder test ((C), affected forelimb, (D), affected hind-limb) are shown. There were no significant impairments on the CTT ($p = 0.214$), but significant impairments on NDS and ladder test were found ($p < 0.001$). Use of TH did not improve NDS scores ($p = 0.120$) or mitigate the forelimb walking impairment ($p = 0.284$), but TH did worsen the hindlimb impairment (*$p = 0.010$). In most cases there were no baseline differences (pre-surgery), and scores were in the typical range for normal rats. CTT, corner turn test; NDS, neurological deficit scale. NORMO n = 13-14, HYPO n = 10-11.

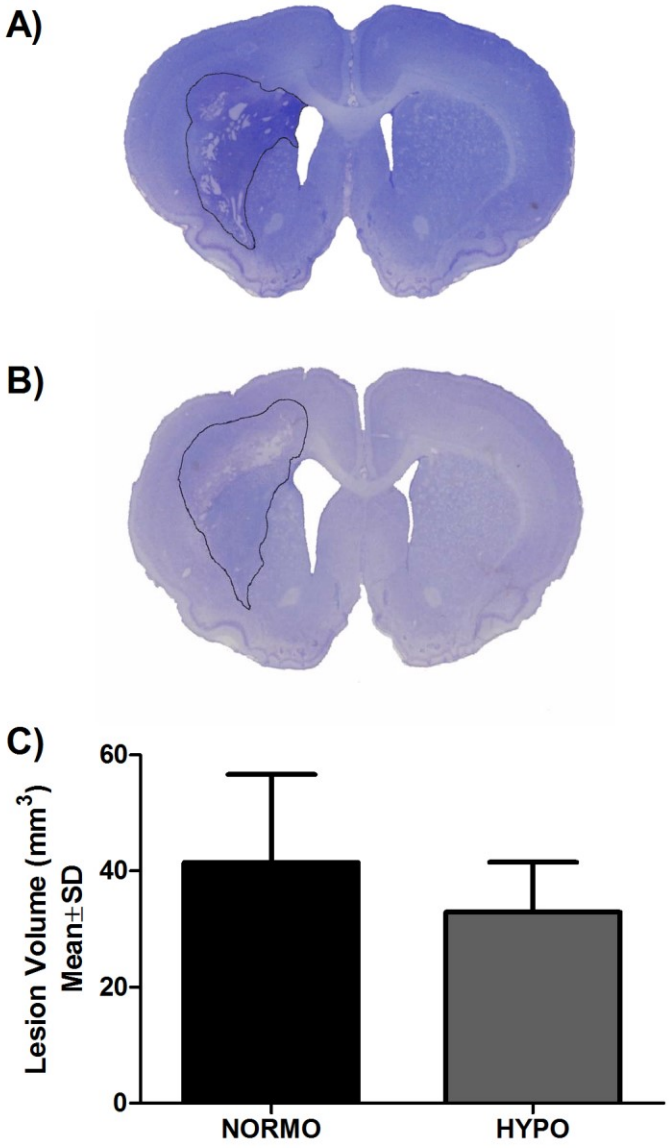

Fig. 4. Infusion of $FeCl_2$ caused significant tissue loss largely within the striatum of both (A) NORMO (n = 14) and (B) HYPO (n = 11) animals (line demarcates the area of damage). (C) The volume of injury was not mitigated by TH at 7 days survival (p = 0.110).

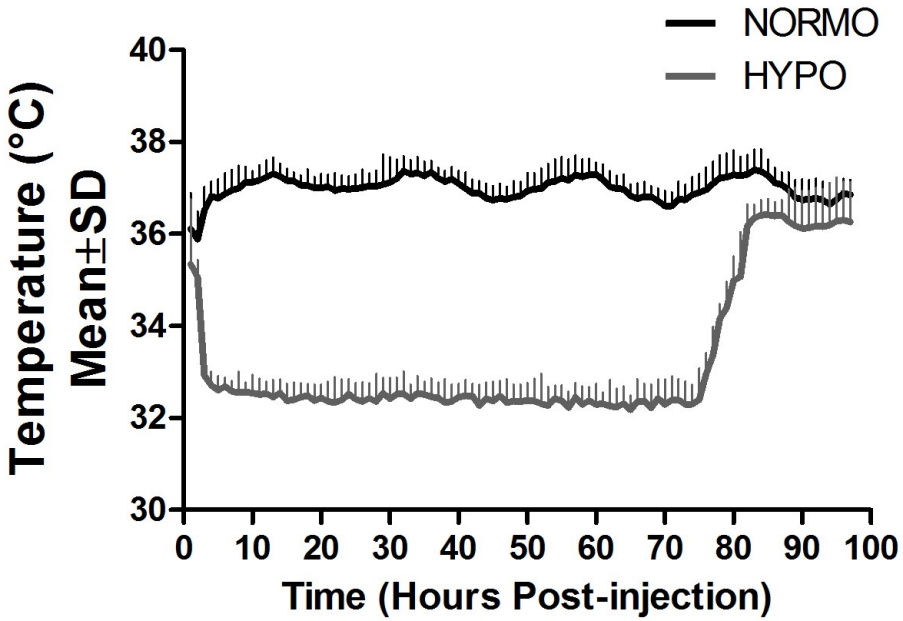

Fig. 5. Core temperature (°C) was measured through implanted telemetry probes (two samples per minute with data averaged every hour). Whole-body hypothermia was initiated at 1 hour after $FeCl_2$ infusion and typically kept to within 0.5°C of the target temperature. These data from experiment 4 are representative of all experiments. Baseline temperature data (not shown) were normal in all cases. n = 16/group.

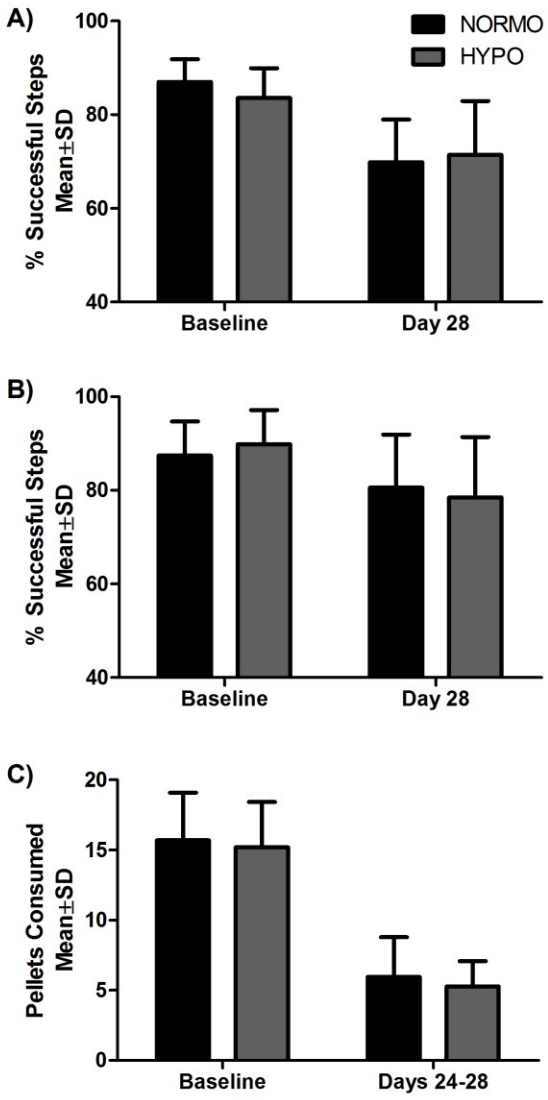

Fig. 6. Significant and lasting behavioral deficits ($p < 0.001$) occurred weeks after $FeCl_2$ infusion for the ladder ((A), affected forelimb, (B), affected hindlimb; % stepping success) and Montoya staircase tests ((C), reaching success for obtaining food pellets). There was no protective effect of TH ($p \geq 0.193$). In all cases, pre-surgery baseline scores were normal and not different between groups. NORMO n = 14-16, HYPO n = 12-16.

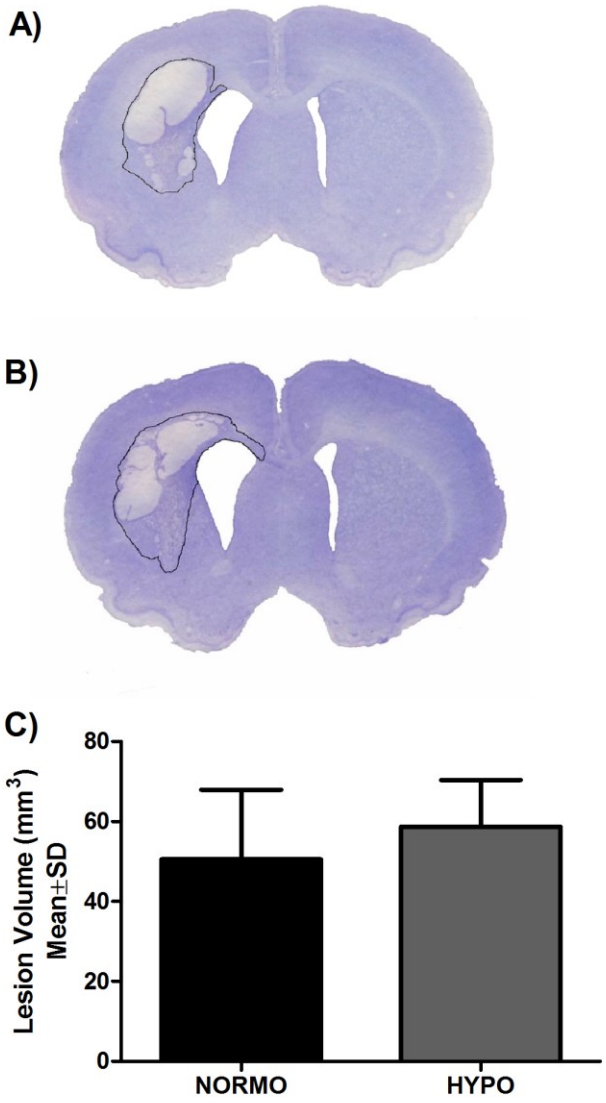

Fig. 7. Infusion of $FeCl_2$ caused damage largely to the striatum of (A) NORMO and (B) HYPO animals as measured at 28 days (*line* demarcates area of damage). (C) Use of TH did not mitigate injury ($p = 0.132$). Note that the quantification of injury also takes into account atrophy (e.g., ventricular dilation – shown). n = 16/group.

Chapter Four

Examining Potential Side Effects of Therapeutic Hypothermia in Experimental Intracerebral Hemorrhage

4.1 Introduction

Intracerebral hemorrhage (ICH), caused by a ruptured blood vessel in the brain, has a high mortality rate and often causes lasting impairments.[1,2] There are no neuroprotective therapies for ICH with treatment relying on medical management and rehabilitation. Primary damage from an ICH is due to mechanical destruction as the blood tears through tissue creating a mass effect, which can sometimes increase intracranial pressure (ICP). This damage occurs quickly with bleeding ceasing within the first 3 hours in most patients.[3] Secondary damage occurs over hours and days later. Mechanisms contributing to secondary injury include inflammation, edema, raised ICP, oxidative stress, and hematoma-related factors such as thrombin production and iron release from degrading erythrocytes.[4-6] Researchers target these secondary mechanisms because their protracted nature allows for later interventions compared to primary injury. Several drugs targeting iron-induced injury (e.g., iron-chelators) and/or hematoma resolution have been studied preclinically, but to date there has been no successful clinical translations.

Therapeutic hypothermia (TH), reducing body and/ or brain temperature, typically in the range of 32-35°C, is considered a gold standard neuroprotectant. It is clinically approved to treat cardiac arrest[7] and neonatal hypoxic–ischemic encephalopathy.[8] The success of TH is likely due to its multifaceted effect on neurodegenerative processes such as inflammation, blood brain barrier (BBB) damage, oxidative stress, edema, and raised ICP.[9] Many of these mechanisms of injury are common to other injuries. As such, TH has been the focus of preclinical and clinical research for traumatic brain and spinal cord injury, as well as ischemic and hemorrhagic stroke, among others.[10-12] In fact, there is abundant preclinical evidence supporting the use of TH for treating ischemic stroke. Studies from different labs, using multiple models (including co-

morbidities), and species, have found TH to reduce cell death and improve behavioral deficits after experimental ischemic stroke.[9] To date, phase II clinical trials suggest that TH is feasible and safe for treating ischemic stroke,[13,14] and TH is currently being investigated in phase III clinical trials.[15]

Despite the overlap of neurodegenerative mechanisms between ischemic and hemorrhagic stroke, the preclinical evidence on using TH to treat ICH is mixed. In ICH, TH does generally mitigate several mechanisms of injury such as inflammation, BBB damage, edema, and raised ICP.[16-20] While some TH studies have found neuroprotection and attenuated behavioral impairments after ICH,[17,18,21,22] others have failed to find those effects.[16,19,23] Such inconsistency is likely due to a number of factors. For instance, some studies have used the collagenase model (collagenase damages blood vessels to cause a bleed), whereas others have infused blood.[16,18,19,22,24] Another factor may be treatment timing as beginning TH early can aggravate bleeding, at least in the collagenase model.[22,25] Despite using an appropriate treatment delay and the same model, the beneficial effects of TH vary among studies in our lab.[22,24] Clinically, small trials have suggested that TH is effective in lowering high levels of edema and mortality,[26] as well as lowering inflammation[27] and improving cerebral blood flow and recovery.[28] The clinical studies conducted so far have been small, used historical controls, and/or participants were typically limited to those with large hemorrhages who are at risk of developing lethal levels of ICP. There may be many complications or side effects associated with using TH to treat ICH that supersede the beneficial effects. Aside from applying TH too early, no TH studies have investigated potential complications specifically associated with treating ICH.

Inflammation is a crucial mechanism for mitigating the toxic components of the hematoma. Microglia and infiltrating macrophages are essential for containing the hematoma and

limiting exposure of the peri-hematoma tissue to blood and clotting factors.[29,30] As well, these cells are essential in hematoma resolution and storing the iron originating from erythrocytes.[31] As TH is a potent anti-inflammatory treatment, we hypothesized that TH impairs endogenous mechanisms for hematoma containment and other aspects related to hematoma resolution. In order to assess this, we completed three experiments using a collagenase model of ICH in rats. First, we evaluated the spread of iron into the surrounding parenchyma, then the rise in non-heme iron from three to seven days post-ICH, and finally the amount of blood left in the brain at one, three, and seven days. We used a collagenase model as this causes more ongoing bleeding than the whole-blood model and is reasonably consistent with greater impairments and a longer period of ongoing cell death.[32] The greater bleeding is essential as it can be an important complication of using TH to treat ICH.[22,25]

4.2 Methods

Animals and experimental conditions

All procedures were in accordance with the Canadian Council on Animal Care under the approval of the University of Alberta's Biosciences Animal Care and Use Committee. One hundred and sixteen male Sprague-Dawley rats from the University of Alberta's Science Animal Support Services colony (250-300g ~10 weeks old) were housed in polycarbonate cages with wood chip bedding in a temperature and humidity-controlled room on a 12-hour light cycle. Animals were given *ad lib* access to water and food (Rodent Chow, Lab Diet) and were randomized to either normothermia (NORMO) or TH (HYPO) groups by a random number generator. Group sizes were determined based on our previous experiments using the same techniques (e.g., for hemoglobin levels after ICH the average standard deviation (S.D.) was ~15 after ICH,[25] and for non-heme iron the SD was 1.2 on day 3 after ICH3)[38]. From that we roughly

obtain estimates for effect sizes and variability with the goal of having 80% power to detect those effects. Most assessments were analyzed blindly except when animals were euthanized cold for non-heme iron levels and hematoma volume analysis at days 1 and 3 post-ICH. In those cases, the experimenter easily identified the cold animals by touch. These experiments are in compliance with the ARRIVE guidelines.[33] Three experiments were completed for this study. First, we evaluated whether TH affected the spread of iron from the hematoma three days post-stroke (n=8/group) as well as the number of inflammatory cells. Second, we studied whether TH impacted the release of iron from heme by comparing non-heme iron levels at three and seven days post-stroke (n=10/group). In this experiment, animals euthanized on day 7 also underwent behavioral testing at baseline (i.e., prior to any surgery) and on day 7 just prior to euthanasia. Last, we evaluated the effect on TH on hematoma volume at one, three, and seven days post-ICH (n= 10/group). Animals in this experiment that were euthanized on day 7 also underwent behavioral testing at baseline and day 7 just prior to euthanasia.

Temperature probe surgery

Four to five days prior to ICH surgery rats had a core temperature telemetry probe (calibrated within 0.2°C, TAT10TA-F20 or F40 Transoma Medical; St. Paul, MN) implanted in their peritoneal cavity.[19] Briefly, rats were anesthetized with isoflurane (4% induction and 1.5-2% maintenance, with 60% N_2O and balance O_2) and an incision was made into the abdominal cavity and a sterilized probe was inserted. The wound was sutured and Marcaine was used as a local anesthetic (~0.1 mL infiltrating the wound area). Rats were placed in an individual clean cage and monitored after they awoke from anesthesia.

ICH surgery

The collagenase-induced ICH model is commonly used in rodents.[32,34] A rectal probe measured temperature during surgery and normothermia was maintained via a water blanket. Rats were anesthetized with isoflurane and a midline incision was made along the scalp. Using a stereotaxic frame, the skull was balanced and a hole, 0.5 mm anterior and 3.5 mm lateral to Bregma, was drilled. A 26-gauge Hamilton syringe was lowered 6.5 mm from the surface of the skull and 0.14 U of type IV-S collagenase (in 0.7 μL of sterile saline) was infused over 5 min. The needle was left in place for an additional 5 min to prevent backflow and then was slowly withdrawn. A metal screw was used to plug the drilled hole. The scalp was sutured and Marcaine was applied (~0.1 mL infiltrating the wound area). Rats were placed in a clean cage and their temperature was monitored while recovering from anesthesia. Rats were monitored frequently to ensure that they were eating and drinking after surgery and otherwise behaving as expected (at least five observations per day until euthanasia). Body weight was taken daily. Additional food was provided for up to four days after ICH in an effort to minimize weight loss (e.g., mixture of peanut butter with sunflower seeds and moistened rodent chow).

Temperature monitoring and control

All animals were confirmed to have normal baseline temperatures (average of ~37.5°C). The rats' cages sat on telemetry receivers (RPC-1, Transoma Medical) and body temperature was sampled every 30 seconds via A.R.T. 2.3 telemetry software (Transoma Medical). After ICH surgery, NORMO rats were only monitored, while HYPO rats' temperature was regulated as follows. Twelve hours post-collagenase injection, TH (33.0°C±0.5) was initiated over an hour and maintained for 72 hours or until euthanasia. The delay was to ensure that TH did not worsen bleeding, which was found with earlier cooling in this model.[22] HYPO rats that were euthanized on day 7 in the second and third experiments had 6 hours of rewarming (0.5°C/hour). A servo-

regulated system consisting of a fan, water mister, and heat lamp was used to induce and maintain hypothermia in awake and freely moving animals as has been routinely used in our lab.[22,35,36]

Histology

Seventy-two hours after ICH, the rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and trans-cardially perfused with 0.9% saline followed by 10% formalin. The brains were removed and later cryostat sectioned (50 μm) using a Teflon-coated blade to prevent metal contamination. The series of sections with the greatest hematoma area were used for rapid-scanning X-ray fluorescence imaging (XFI), cresyl violet, and Perls' Prussian Blue staining. The cresyl violet sections were used to define the hematoma border; however, due to edema and blood, we did not attempt to determine lesion volume assessment at day 3. The Perls' stain was used to quantify the number of macrophages/microglia in the peri-hematoma and surrounding tissue based on iron labeling and morphology as has been previously done.[19,37] Cells were counted in one section per animal at the level of maximal injury, as has been previously done and found to detect a significant effect of hypothermia.[19]

X-ray fluorescence imaging

This imaging technique has been previously used to quantify and localize elements, with iron of most interest, in a coronal section of tissue after ICH.[38] This technique is highly sensitive and the same section of tissue can be used for other histological procedures following imaging (i.e., cresyl violet).[38-40] Briefly, sections used for XFI were placed on plastic metal-free cover slips (Thermanox; Rochester, NY) and imaged at the Stanford Synchrotron Radiation Light Source on beamline 10-2 at a 50-μm resolution and a 50-ms dwell time. The incident energy of

the X-ray beam was 13 keV and the slides were mounted 45° to the incident X-ray beam and 45° to the detector. Signal strengths of calibrations standards (Micromatter Technologies Inc.; Surrey, BC) were compared to the sample signals and used to quantify iron concentrations. Images were analyzed with Sam's Microanalysis Toolkit.[41]

Non-heme iron assay

The non-heme iron assay was used to determine whether TH influenced the rate of iron release from heme at survival times of three and seven days after ICH. Rats were deeply anesthetized with 4% isoflurane, decapitated, and a 6 mm thick section of both fore-brains were taken (2 mm anterior and 4 mm posterior to the collagenase injection site). The cerebellum served as control. Each forebrain and the cerebellum were homogenized with dH_2O and iron was released from proteins, except heme, by mixing the sample with a solution of 1 N HCl and 10% trichloroacetic acid in dH_2O and heating to 95°C. The samples were centrifuged and the supernatant was collected and reacted with a ferrozine chromagen solution and compared to a standard curve to determine iron concentration.[38,42]

Behavioral testing

In the second experiment, the corner turn test (CTT) and neurological deficit scale (NDS) were used to evaluate deficits in rats surviving to seven days post-ICH. Animals were allowed to acclimatize to the testing room 30 minutes prior to behavioural testing to reduce stress. In the third experiment, the NDS was used on day 7. In both experiments, baseline behavioral testing occurred prior to temperature probe implantation and all testing occurred during the light cycle. Both behavioral tests are sensitive to striatal injury.[43,44] However, the CTT was not used in experiment three, as the test is tedious and did not add any information that the NDS did not

already provide. The CTT has two walls (41 cm height by 30.5 cm width) placed at a 30° angle with a 0.5 cm gap. At baseline, the direction a rat turns coming out of the corner was averaged over two days (10 trials/ day), and rats that had a turning bias (<30% in one direction) at baseline were excluded from analysis for this test. At testing, rat's turning preference was determined by taking an average of 10 trials. The NDS is a combination of several simple motor tasks (hind-limb retraction, contralateral forelimb flexion, bilateral fore-paw grasp, beam walking, and spontaneous circling) and is the most commonly used test in experimental ICH research.[45] Each task ranges from 0 to 3 in score, except the contralateral forelimb flexion that ranges from 0 to 2. A score of fourteen denotes maximum impairment.

Hematoma volume

Blood volume was measured using a spectrophotometric hemoglobin assay and compared to a standard curve of known blood volumes.[25,46,47] Rats were euthanized one, three, or seven days post-ICH via deep isoflurane anesthesia and decapitation. The hemispheres were separated and homogenized in double distilled water (1:4 w:v ratio), incubated on ice, and then centrifuged at 15,800 *g* for 35 min. Aliquots of the supernatant were reacted with Drabkin's solution and the absorption values were obtained (measured at 540 nm; Model 4001/4; Thermo Fisher Scientific) and compared to the standard curve. Hematoma volume was calculated as injured forebrain (IPSI) – contralateral forebrain (CONTRA) blood volume. The latter was used as an estimate of the amount of blood present within the vasculature.

Statistical analysis

All data are expressed at mean ± S.D. except the NDS scores, which are presented as raw scores and medians ± interquartile range (I.Q.R.). All data were analyzed using analysis of

variance (ANOVA) or independent t-tests (SPSS v.21; SPSS, Inc.) except the NDS scores, which were analyzed via the Mann–Whitney *U* and Wilcoxon tests. Also, we correlated the XFI analysis of distance and iron levels, and also the NDS and hematoma values, providing the Pearson r and p values. A Fischer's exact test was used for the bleeding data of experiment three. The Levene's test was used to test for homogeneity of variance and when there was a significant effect we used t-tests that did not assume equal variances. When a significant effect was detected, a post hoc effect size (Cohen's d) was calculated (G × Power v3.1.3; Univerität Kiel, Germany). Significance was at $p < 0.05$.

4.3 Results

Experiment one: Spread of iron and number of Perls' positive cells

There were no exclusions or mortality in this experiment. We found TH significantly reduced the number of Perls' positive cells in the HYPO group ($p = 0.012$, equal variances not assumed; Fig. 1(A) to (C)). The size of the effect was large ($d = 1.5$).

Overall, using XFI, we found a moderate but significant relationship with iron levels declining with distance from hematoma ($r = 0.333$, $p = 0.007$, Fig. 2(A) and (B)). There was no significant difference between NORMO and HYPO in the average amount of iron ($p = 0.825$, Fig. 2(C)) or distance from hematoma our samples were taken from ($p = 0.674$, Fig. 2(D)). There was a significant increase in total iron levels in the injured hemisphere ($p < 0.001$ vs. CONTRA hemisphere) but no effect of group ($p = 0.567$) and no interaction ($p = 0.419$). Thus, while TH caused a significant reduction in inflammatory cells, this did not affect the spread of iron or the total amount of iron in the injured hemisphere.

Experiment two: Non-heme iron levels and behavioural impairments

In this experiment, there were two exclusions and two mortalities, with the latter cases also being excluded from our analysis. One animal was excluded from NORMO-Day 3 for a faulty probe and one from HYPO-Day 7 for being unable to self-regulate body temperature after rewarming. One animal spontaneously died during TH from HYPO-Day 7 and one animal from NORMO-Day 7 died during ICH surgery. The baseline temperatures in all groups were normal (data not shown) and Figure 3(A) and (B) shows the temperature data post-ICH in a subset of animals (n=9 for each NORMO group and n=6 for each HYPO group). These data are representative of the other experiments in this study.

Behavioral impairments were observed on day 7 post-stroke. For the CTT (Fig. 4(A)), there was a significant time effect ($p < 0.001$) but no group effect ($p = 0.747$) or interaction ($p = 0.937$). As well, the NDS (Fig. 4(B)) showed a significant impairment on day 7 ($p < 0.001$ vs. baseline) but no significant group effect at either time ($p \geq 0.316$).

There was an increase in non-heme iron levels in the IPSI hemisphere between day 3 and day 7 (Fig. 5) as there was a significant time effect ($p < 0.001$) but no group ($p = 0.479$) or interaction ($p = 0.662$) effects. The size effect of this increase was large ($d = 2.3$). As expected, in the CONTRA hemisphere, there were no effects of time ($p = 0.071$), group ($p = 0.882$) or interaction ($p = 0.413$). Likewise, there were no time ($p = 0.789$), group ($p = 0.420$), or interaction ($p = 0.990$) effects for cerebellum non-heme iron levels. TH had no effect on the increase of non-heme levels that occurred between three and seven days post-stroke or the behavioral impairments caused by ICH.

Experiment three: Hematoma volume and behavioural impairments

One animal was excluded from HYPO-Day 3 due to experimenter error and one animal from NORMO-Day 7 died during ICH surgery. The data from these animals were not included in our analyses. There were significant impairments detected with NDS at 7 days post-stroke ($p < 0.001$ vs. baseline; Fig. 6) and no difference between groups at baseline ($p = 0.424$). However, TH caused greater impairments ($p = 0.011$) and the size of the effect was moderate ($d = 0.7$). As there was a slightly different pattern of baseline NDS scores between groups, we also analyzed the difference score (day 7 – baseline), which also showed that TH worsened impairment ($p = 0.013$).

There was a significant difference in hematoma volume (Fig. 7(A)) as there were significant time ($p = 0.004$), group ($p = 0.014$), and interaction ($p = 0.046$) effects. There was no difference between HYPO and NORMO at day 1 or 3 ($p \geq 0.264$), but there was significantly more blood in the HYPO-Day 7 group compared to the NORMO-Day 7 group ($p = 0.032$, equal variances not assumed). There was a bimodal distribution of hematoma volume in the HYPO group euthanized on day 7 with 40% of the animals having a more than quadrupling in hematoma volume. This difference between NORMO and HYPO on day 7 was large ($d = 1.1$). The proportion of animals in this group that landed outside a cut off (i.e., three standard deviations of all other groups) was different than all other groups ($p = 0.002$, Fischer exact test). In fact, only one animal from all the other groups ($n = 48$) barely fell out of this range, while 4 of 10 from HYPO-Day 7 did. There were no time ($p = 0.380$), group ($p = 0.589$), or interaction ($p = 0.677$) effects for the CONTRA hemisphere blood volumes ($p \geq 0.380$). Thus, while TH did not influence bleeding during treatment, there was considerable rebleeding in a subset of animals post-treatment. Visual inspection of the injured hemispheres of these animals with the larger bleeds showed an obviously greater amount of blood that was fresher in nature (i.e. bright red vs.

rust colored of an older bleed). Experimenters were blinded to treatment of all animals euthanized on day 7.

Although NDS scores were significantly worse in the HYPO group on day 7 post-ICH (vs. NORMO), the NDS scores did not significantly ($r = 0.314$, $p = 0.190$) predict hematoma volume (Fig. 7(B)).

4.4 Discussion

Although our data do not confirm our hypothesis that the anti-inflammatory effects of TH impacts hematoma containment and components of hematoma resolution, a different complication of TH treatment was discovered. Post-treatment, 40% of HYPO animals had a large increase in hematoma volume, while during treatment there were no group differences. Whether this bleeding occurred during rewarming or over subsequent days is unclear. This treated group also had modestly worsened behavioral impairments. Our data confirm other work showing that TH is a potent anti-inflammatory,[17,19,48] as we saw a 35% decrease in Perls' positive cells (i.e., microglia/macrophages). We also found a significant relationship between distance and iron levels, with higher iron levels closer to the border of the hematoma. There was no effect of TH. On day 3, there was no difference in non-heme iron levels between the injured hemisphere and the control structure (i.e., cerebellum) suggesting that the majority of iron is still contained in heme at that time. However, from three to seven days post-ICH, we found an increase in non-heme iron levels, which TH did not influence. This increase in non-heme iron matches previous work.[38] There was no effect of TH on behavioral deficits in this experiment. Overall, these results suggest that while TH did not influence endogenous mechanism of hematoma containment and clearance, there was occasionally a post-treatment worsening of

bleeding. That latter effect should be considered in future animal research and is a significant concern for clinical use of TH for ICH.

Although not part of our initial hypothesis, the most interesting and concerning result of this study is the influence of TH on bleeding post-treatment, since the hematoma volume is a critical predictor of outcome and survival.[49,50] Previous studies show that TH applied too early can aggravate bleeding,[22,25] which we successfully avoided by delaying treatment, as we have previously done.[24] As there were no group differences in hematoma volume at 1 and 3 days post-ICH, the increase seen on day 7 is likely due to rebleeding either during rewarming or over the following days. There are a number of reasons to support that this effect was not due to chance or experimenter error. First, all the animals with a large bleed occurred in one group and all of these animals are outliers beyond the combined three standard deviations of the other groups. As well, animals were randomized to groups and the experiment was performed in eight cycles over several months with three of these cycles having animals with large bleeds. Two experimenters conducted the hemoglobin assay throughout the study and both had instances of large bleeds. Finally, control samples from each animal (i.e., contralateral hemi-sphere and cerebellum – data not shown) were normal in all animals. All of this suggests that the increase in hematoma volume is a genuine effect of TH. Of course, future studies will need to replicate these findings and to precisely determine the incidence rate, currently at 40%, which will require large group sizes to ensure adequate statistical power. As well, it is important to identify factors that influence the incidence and magnitude of rebleeding complications, such as, potentially, the initial insult severity and various treatment parameters (e.g., depth and duration of cooling).

Importantly, the intermittent occurrence of rebleeding likely contributes to the lack of a consistent neuroprotective effect in animal studies. Although not all treated animals have this

increase in hematoma volume, when it occurs the effect can be substantial. In a group of 8-10 animals, the occurrence of one or two aggravated bleeding events of even half the current magnitude could easily counteract the ability to statistically detect a neuroprotective effect of cooling. Many of the studies looking at neuroprotection have histological and behavioral endpoints weeks after the initial bleed. At these times, the hematoma has resolved and it would be impossible to determine that bleeding was worsened. Even when behavioral outcome is measured at a week, as in this study, there is only a small and inconsistent worsening of impairments that can be easily missed. Although behavioral tests, such as the NDS, are commonly used in ICH studies,[45] they often do not reliably detect even substantial differences in lesion size.[43,44] Likewise, in this study, the NDS scores did not significantly predict hematoma size. Therefore, the impact of rebleeding can be overlooked both histologically and functionally in rodent studies when one does not directly measure hemoglobin levels. Even the non-heme iron assay may not pick up delayed bleeding if it occurs before hemoglobin has broken down and liberated its iron, which likely explains why we did not find an increase in non-heme iron levels at day 7 in experiment two. It is also possible that rebleeding did not occur in that study, as our last study suggests that it is an intermittent problem, and thus by chance fewer or no complications may have occurred in the non-heme experiment.

Clinical trials evaluating the use of TH for ICH have suggested benefit, which is likely due to a decrease in life threatening levels of edema where ICP is substantially increased. Only one trial evaluated hematoma volume post-treatment of TH (without hematoma evacuation) and found no increase, although this was assessed early after rewarming.[26] An increase in bleeding post-treatment may be missed if quantifying hematoma volume too early, as we did not notice an effect when cooling was being maintained. There are also protocol differences between this trial

and the current study, where cooling was milder (~35°C vs. 33°C) and rewarming was slower (0.5°C/24 hour vs. 0.5°C/hour) in the trial. As well, there may be physiological differences between humans and rats during and after TH (e.g., changes in BP). However, the impact of TH on ICP after large ICH seen in this trial has also been seen in rats.[20] While a slow increase in hematoma volume may not cause mortality, as is the case in this experiment, the increase may negate the long-term impact of TH on outcome in affected patients. As well, in patients with small to moderate sized hematomas where edema is not typically life threatening, the complications of TH may counteract any beneficial effects.

There are a number of potential causes for this unexpected increase in hematoma volume such as changes in BP, cerebral blood flow, or stress response, which we did not presently measure. Such events might be especially likely to cause bleeding because of significant BBB damage that continues to worsen over days after collagenase infusion.[32] In that study, we used magnetic resonance imaging to quantify gadolinium extravasation in a comparable collagenase model. It is not clear from our present data whether late rebleeding started during rewarming or after normothermia was reached. Unfortunately, timing will have to be determined through the use of additional survival times because repeated imaging in animals would be technically difficult in rats subjected to prolonged cooling (e.g., ensuring a continuous cooling protocol) and contraindicated in some cases (e.g., if animals have implanted electronics to sense temperature). Future work will investigate the mechanism(s) of this hematoma increase, but the intermittent nature of this effect, for whatever reasons, increases the difficulty of this work and will require large sample sizes. However, as larger scale clinical study is currently underway, this complication is important to understand, even if it is an infrequent event.

The inflammatory response after an ICH is complex as this response can be both harmful and beneficial to brain tissue. We had hypothesized that the anti-inflammatory effect of TH may decrease the harmful aspects of inflammation while also impeding beneficial functions. There are several mechanisms through which the inflammatory response causes damage. Inflammatory induced cytotoxic edema and cell death occurs through exposure to cytokines and oxidative stress.[51] Vasogenic edema is caused by disrupting the BBB through increased secretions of matrix metalloproteinases.[51]Treatment with minocycline, a common anti-inflammatory, has shown that it lowers edema and reduces injury after ICH.[52,53] Experimentally depleting neutrophils also inhibits microglia and macrophage proliferation and reduces BBB disruption, brain damage, and impairment.[54,55] In contrast, others have demonstrated the importance of inflammation especially after bleeding. For example, Zhao and others[30] suggest that the transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ) is beneficial by increasing phagocytosis in microglia and macrophages, which is essential in hematoma resolution. These studies highlight the complexity of post-ICH inflammation.

The influence of TH on inflammation is known and contributes to one of the main adverse events associated with TH, pneumonia.[56] Use of TH lowers pro-inflammatory cytokines after ICH in humans and animals,[18,27,57] while anti-inflammatory cytokines are increased[18] and inflammatory cell proliferation is decreased.[17,19] The role of TH on phagocytosis is not well studied after ICH. In cell culture, after rewarming TH decreases microglia proliferation but increases microglia activity (i.e., ATP consumption) and phagocytosis behavior.[58] In cerebral ischemia, Kawabori and others[59] found that TH increased the proportion of cells expressing of triggering receptor expressed on myeloid cells-2 (TREM-2), a receptor important for microglia phagocytosis. Thus, TH may not impact the inflammatory response's abilities to contain iron or

resolve the hematoma because phagocytosis is being upregulated. However, further studies on ICH are needed to test this hypothesis.

With XFI we were able to both spatially map and quantify total iron levels, which is superior to biochemical assays. Unfortunately, XFI does not distinguish the form of iron or whether it is bound to protein (e.g., heme and ferritin) or contained within a cell (e.g., microglia). The quantity of iron observed outside the hematoma was significantly above contralateral levels. Whether that peri-hematoma iron originated from hemoglobin breakdown or from microglia containing iron infiltrating or leaving the injured site is unclear. We did not find an effect of TH, but we may have missed effects in the tissue immediately bordering the hematoma. It is not easy to precisely determine that border (e.g., confounds of peri-hematoma edema). Thus, regions of interest were randomly sampled in the injured hemisphere at varying distances from the hematoma, but none were directly adjacent to the border. Thus, a border was determined as best as technically possible and regions were sampled near the border. For future studies, we recommend Raman imaging of hemoglobin in conjunction with XFI as a way of better defining that immediate border zone.[60]

There appears to be a discrepancy between the increase in non-heme iron from three to seven days and the lack of a decrease in hematoma volume (i.e., hemoglobin) in the normothermic groups for the same times. Both assays indirectly evaluate hematoma resolution and measure two distinct components of the hematoma, a metal (i.e., iron not contained by heme) and a protein (i.e., hemoglobin). There are a couple of explanations for this discrepancy. First, since this experiment was conducted, new data from our lab (Williamson et al. unpublished data) show that this widely used hemoglobin assay is not specific to hemoglobin. While the majority of the absorbance is for hemoglobin, this assay also detects two hemoglobin breakdown

products, hemin and to a lesser extent bilirubin. The added absorbance of hemin and bilirubin detected by this assay will partially mask the reduction in hemoglobin levels over time. Also, as edema resolves from day 3 to 7, this decrease in water content make it appear that there is an increase in tissue concentration of non-heme iron.[38] Given that there was a substantial non-heme iron increase, edema resolution is not likely the only factor as there is likely some release of iron from hemoglobin. As well, the increase in hematoma volume in the four treated rats who were rewarmed cannot be explained by heme and bilirubin contributing to the absorbance reading of the hemoglobin assay as these breakdown products have less absorbance than hemoglobin.

There was a significant worsening of behavioral impairments by TH in experiment three but not in experiment two. The worsening of behavioral impairments in the third experiment was significant but small, and it may not have much biological significance. More sensitive tests are needed to determine this. It is not clear why there was no worsening of deficits in HYPO rats in experiment two, but it might be due to test insensitivity or fewer animals experiencing rebleeding. There is also a slight difference in baseline scores between experiments two and three, which is either due to chance, or the subjective nature of NDS as different experimenters conducted the behavioral testing for these two experiments. Fortunately, animals were randomized to treatment condition in all cases, experimenters were blinded, and there was still a significant impairment after ICH in both experiments. Although possible, it is not likely that the collagenase insults varied all that much among studies as this surgical procedure is highly standardized and we used the same batch of collagenase for all of the work.

In summary, the increase in hematoma volume after TH is a pressing concern for its clinical use in ICH and may help explain the lack of consistent neuroprotection in animal studies. Future studies must evaluate this rebleeding problem and investigate possible mechanisms while

varying treatment and model parameters. Contrary to our hypothesis, the decrease in inflammatory cells caused by TH does not appear to impair the brain's ability to contain iron or influence the hematoma-related factors. This may be due to increased phagocytosis behavior caused by TH but further investigation is needed to study this.

4.5 References

1. Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB. The stroke data bank: Design, methods, and baseline characteristics. Stroke 1988 May;19(5):547-554.

2. Sacco S, Marini C, Toni D, Olivieri L, Carolei A. Incidence and 10-year survival of intracerebral hemorrhage in a population-based registry. Stroke 2009 Feb 1,;40(2):394-399.

3. Davis SM, Broderick J, Hennerici M, Brun NC, Diringer MN, Mayer SA, et al. Hematoma growth is a determinant of mortality and poor outcome after intracerebral hemorrhage. Neurology 2006 Apr 25,;66(8):1175-1181.

4. Hackett MJ, DeSouza M, Caine S, Bewer B, Nichol H, Paterson PG, et al. A new method to image heme-fe, total fe, and aggregated protein levels after intracerebral hemorrhage. ACS Chem Neurosci 2015;6(5):761-770.

5. Testai FD, Aiyagari V. Acute hemorrhagic stroke pathophysiology and medical interventions: Blood pressure control, management of anticoagulant-associated brain hemorrhage and general management principles. Neurol Clin 2008 Nov;26(4):963-ix.

6. Wu X, Stezoski J, Safar P, Bauer A, Tuerler A, Schwarz N, et al. Mild hypothermia during hemorrhagic shock in rats improves survival without significant effects on inflammatory responses. Crit Care Med 2003 Jan;31(1):195-202.

7. Howes D, Gray SH, Brooks SC, Boyd JG, Djogovic D, Golan E, et al. Canadian guidelines for the use of targeted temperature management (therapeutic hypothermia) after cardiac arrest: A joint statement from the canadian critical care society (CCCS), canadian neurocritical care

society (CNCCS), and the canadian critical care trials group (CCCTG). Resuscitation 2016 Jan;98:48-63.

8. Peliowski-Davidovich A. Hypothermia for newborns with hypoxic ischemic encephalopathy. Paediatrics & child health 2012 Jan;17(1):41-43.

9. Yenari MA, Han HS. Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat Rev Neurosci 2012 Feb 22,;13(4):267-278.

10. Darwazeh R, Yan Y. Mild hypothermia as a treatment for central nervous system injuries: Positive or negative effects. Neural Regen Res 2013 Oct;8(28):2677-2686.

11. Marion D, Bullock MR. Current and future role of therapeutic hypothermia. Journal of neurotrauma 2009 Mar;26(3):455-467.

12. Sherman AL, Wang MY. Hypothermia as a clinical neuroprotectant. Phys Med Rehabil Clin N Am 2014 Aug;25(3):519-529, vii.

13. Lyden PD, Hemmen TM, Grotta J, Rapp K, Raman R. Endovascular therapeutic hypothermia for acute ischemic stroke: ICTuS 2/3 protocol. International Journal of Stroke 2014 Jan;9(1):117-125.

14. Hemmen TM, Raman R, Guluma KZ, Meyer BC, Gomes JA, Cruz-Flores S, et al. Intravenous thrombolysis plus hypothermia for acute treatment of ischemic stroke (ICTuS-L): Final results. Stroke 2010 Oct;41(10):2265-2270.

15. van der Worp, H. Bart, Macleod MR, Bath PMW, Demotes J, Durand-Zaleski I, Gebhardt B, et al. EuroHYP-1: European multicenter, randomized, phase III clinical trial of therapeutic hypothermia plus best medical treatment vs. best medical treatment alone for acute ischemic stroke. International Journal of Stroke 2014 Jul;9(5):642-645.

16. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

17. Kawanishi M, Kawai N, Nakamura T, Luo C, Tamiya T, Nagao S. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. J Stroke Cerebrovasc Dis 2008;17(4):187-195.

18. Liu X, Liu X, Jing L, Jing L, Yang M, Yang M, et al. Enhanced neuroprotection of minimally invasive surgery joint local cooling lavage against ICH-induced inflammation injury and apoptosis in rats. Cell Mol Neurobiol 2016 Jul;36(5):647-655.

19. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

20. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

21. Fingas M, Penner M, Silasi G, Colbourne F. Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia. Experimental Neurology 2009;219(1):156-162.

22. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

23. Wu J, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. Brain Res 2002 Oct 25,;953(1-2):45-52.

24. Klahr AC, Dietrich K, Dickson CT, Colbourne F. Prolonged localized mild hypothermia does not affect seizure activity after intracerebral hemorrhage in rats. Ther Hypothermia Temp Manag 2016 Mar;6(1):40-47.

25. John RF, Williamson MR, Dietrich K, Colbourne F. Localized hypothermia aggravates bleeding in the collagenase model of intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2015 Mar 1,;5(1):19-25.

26. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

27. Dohi K, Jimbo H, Ikeda Y, Fujita S, Ohtaki H, Shioda S, et al. Pharmacological brain cooling with indomethacin in acute hemorrhagic stroke: Antiinflammatory cytokines and antioxidative effects. Acta Neurochir Suppl 2006;96:57-60.

28. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

29. Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, et al. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. Stroke 2007 Dec;38(12):3280-3286.

30. Zhao X, Gonzales N, Aronowski J. Pleiotropic role of PPARγ in intracerebral hemorrhage: An intricate system involving Nrf2, RXR, and NF-κB. CNS Neurosci Ther 2015 Apr;21(4):357-366.

31. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: Secondary brain injury. Stroke 2011 Jun;42(6):1781-1786.

32. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

33. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: Reporting in vivo experiments: The ARRIVE guidelines. British Journal of Pharmacology 2010 Aug;160(7):1577-1579.

34. Rosenberg GA, Mun-Bryce S, Wesley M, Kornfeld M. Collagenase-induced intracerebral hemorrhage in rats. Stroke 1990 May;21(5):801-807.

35. Colbourne F, Sutherland GR, Auer RN. An automated system for regulating brain temperature in awake and freely moving rodents. J Neurosci Methods 1996 Aug;67(2):185-190.

36. Klahr AC, Nadeau CA, Colbourne F. Temperature control in rodent neuroprotection studies: Methods and challenges. Ther Hypothermia Temp Manag 2017 Mar;7(1):42-49.

37. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

38. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

39. Zheng W, Nichol H, Liu S, Cheng YN, Haacke EM. Measuring iron in the brain using quantitative susceptibility mapping and X-ray fluorescence imaging. Neuroimage 2013 Sep;78:68-74.

40. Zheng W, Haacke EM, Webb SM, Nichol H. Imaging of stroke: A comparison between X-ray fluorescence and magnetic resonance imaging methods. Magn Reson Imaging 2012;30(10):1416-1423.

41. The microanalysis toolkit: X-ray fluorescence image processing software. Chicago; August 2011.

42. Rebouche CJ, Wilcox CL, Widness JA. Microanalysis of non-heme iron in animal tissues. J Biochem Biophys Methods 2004 Mar 31;58(3):239-251.

43. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

44. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

45. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

46. Choudhri TF, Hoh BL, Solomon RA, Connolly ES, Pinsky DJ. Use of a spectrophotometric hemoglobin assay to objectively quantify intracerebral hemorrhage in mice. Stroke 1997 Nov;28(11):2296-2302.

47. Wowk S, Ma Y, Colbourne F. Therapeutic hypothermia does not mitigate iron-induced injury in rat. Ther Hypothermia Temp Manag 2016 Mar;6(1):23-29.

48. Sheng SP, Lei B, James ML, Lascola CD, Venkatraman TN, Jung JY, et al. Xenon neuroprotection in experimental stroke: Interactions with hypothermia and intracerebral hemorrhage. Anesthesiology 2012 Dec;117(6):1262-1275.

49. Broderick JP, Brott TG, Duldner JE, Tomsick T, Huster G. Volume of intracerebral hemorrhage. A powerful and easy-to-use predictor of 30-day mortality. Stroke 1993 Jul;24(7):987-993.

50. Rost NS, Smith EE, Chang Y, Snider RW, Chanderraj R, Schwab K, et al. Prediction of functional outcome in patients with primary intracerebral hemorrhage: The FUNC score. Stroke 2008 Aug 1,;39(8):2304-2309.

51. Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. Prog Neurobiol 2010 Dec;92(4):463-477.

52. Wu J, Yang S, Hua Y, Liu W, Keep RF, Xi G. Minocycline attenuates brain edema, brain atrophy and neurological deficits after intracerebral hemorrhage. Acta Neurochir Suppl 2010;106:147-150.

53. Xue M, Mikliaeva EI, Casha S, Zygun D, Demchuk A, Yong VW. Improving outcomes of neuroprotection by minocycline: Guides from cell culture and intracerebral hemorrhage in mice. American Journal of Pathology 2010 Mar 1,;176(3):1193-1202.

54. Moxon-Emre I, Schlichter LC. Neutrophil depletion reduces blood-brain barrier breakdown, axon injury, and inflammation after intracerebral hemorrhage. J Neuropathol Exp Neurol 2011 Mar;70(3):218-235.

55. Sansing LH, Harris TH, Kasner SE, Hunter CA, Kariko K. Neutrophil depletion diminishes monocyte infiltration and improves functional outcome after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2011;111:173-178.

56. Andresen M, Gazmuri JT, Marín A, Regueira T, Rovegno M. Therapeutic hypothermia for acute brain injuries. Scand J Trauma Resusc Emerg Med 2015 Jun;23:42.

57. Wagner KR, Beiler S, Beiler C, Kirkman J, Casey K, Robinson T, et al. Delayed profound local brain hypothermia markedly reduces interleukin-1beta gene expression and vasogenic edema development in a porcine model of intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:177-182.

58. Diestel A, Troeller S, Billecke N, Sauer IM, Berger F, Schmitt KRL. Mechanisms of hypothermia-induced cell protection mediated by microglial cells in vitro. Eur J Neurosci 2010 Mar;31(5):779-787.

59. Kawabori M, Hokari M, Zheng Z, Kim JY, Calosing C, Hsieh CL, et al. Triggering receptor expressed on myeloid cells-2 correlates to hypothermic neuroprotection in ischemic stroke. Therapeutic Hypothermia and Temperature Management 2013 Dec 1,;3(4):189-198.

60. Williamson MR, Dietrich K, Hackett MJ, Caine S, Nadeau CA, Aziz JR, et al. Rehabilitation augments hematoma clearance and attenuates oxidative injury and ion dyshomeostasis after brain hemorrhage. Stroke 2017;48(1):195-203.

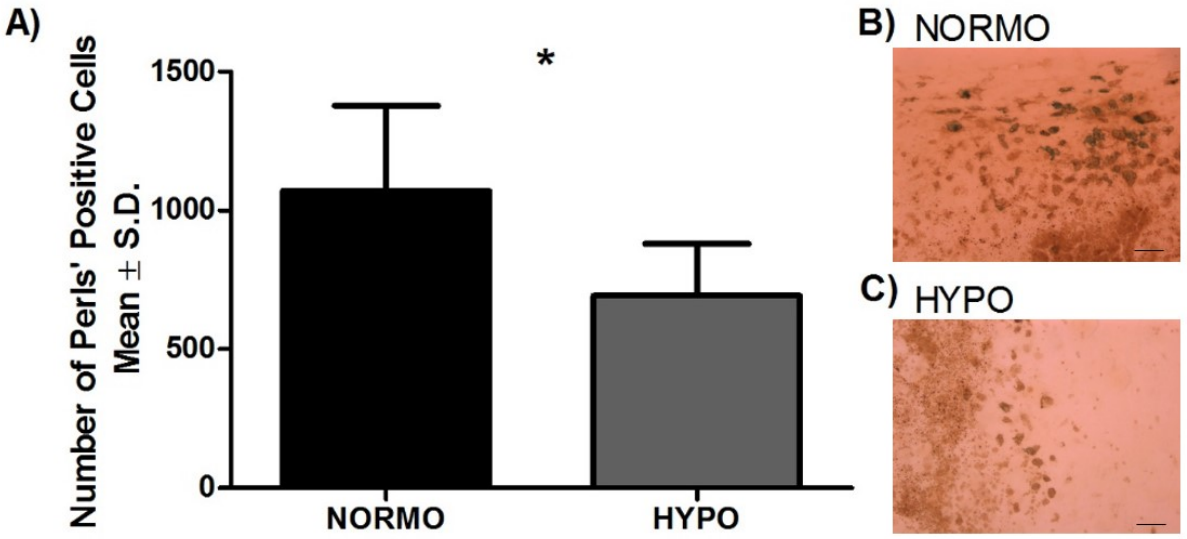

Fig. 1. (A) TH significantly reduced the number of Perls' positive cells by 35% ($p = 0.012$). (B) and (C) are representative images of tissue stained with Perls' Prussian blue for NORMO and HYPO, respectively. Sections from the maximum hematoma were used. n = 8 animals/group; scale bar = 50 mm. *$p < 0.05$.

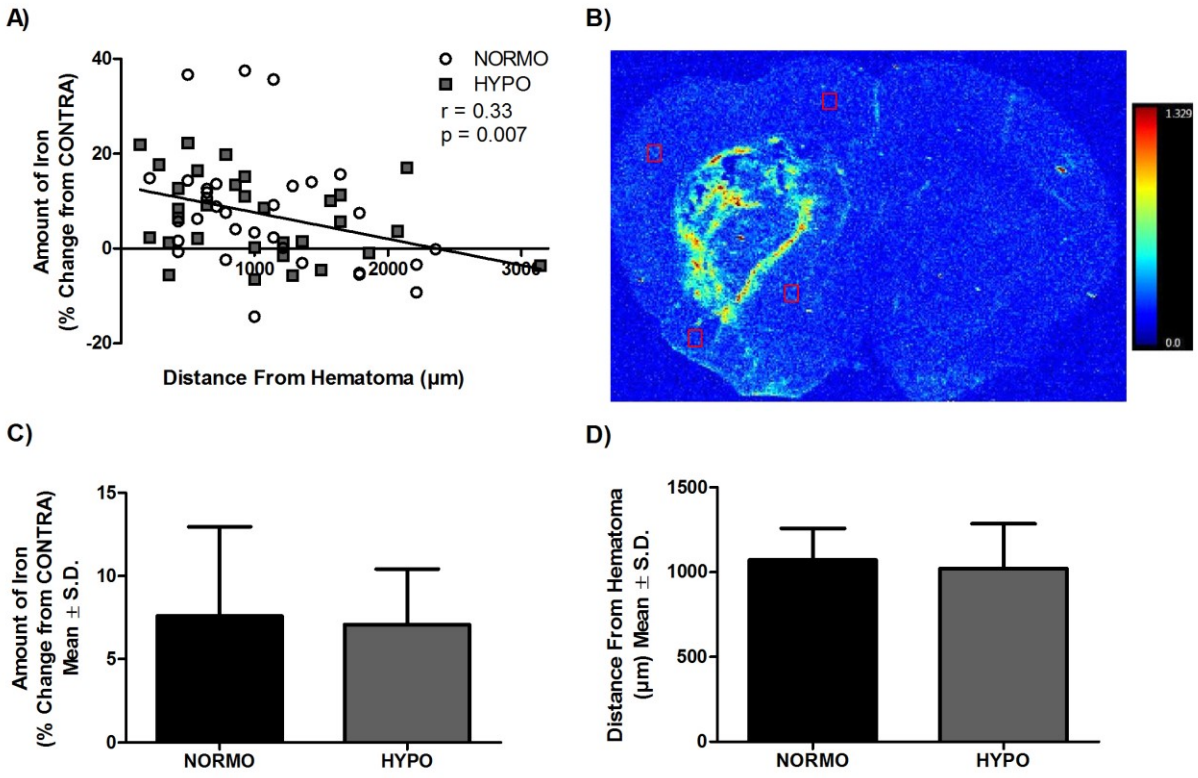

Fig. 2. (A) There was a significant relationship between the amount of iron and distance from the border of the hematoma (r = 0.33 p = 0.007). (B) A representative XFI map showing the amount and location of total iron. The red boxes illustrate representative placements of the regions of interest (four regions/animal). The intensity scale bar ranges from 0.0 to 1.329 µg/cm^2 of iron. (C) The average amount of iron and (D) the average distance of the regions of interest were not different between NORMO and HYPO ($p \geq 0.674$). n = 8 animals/group.

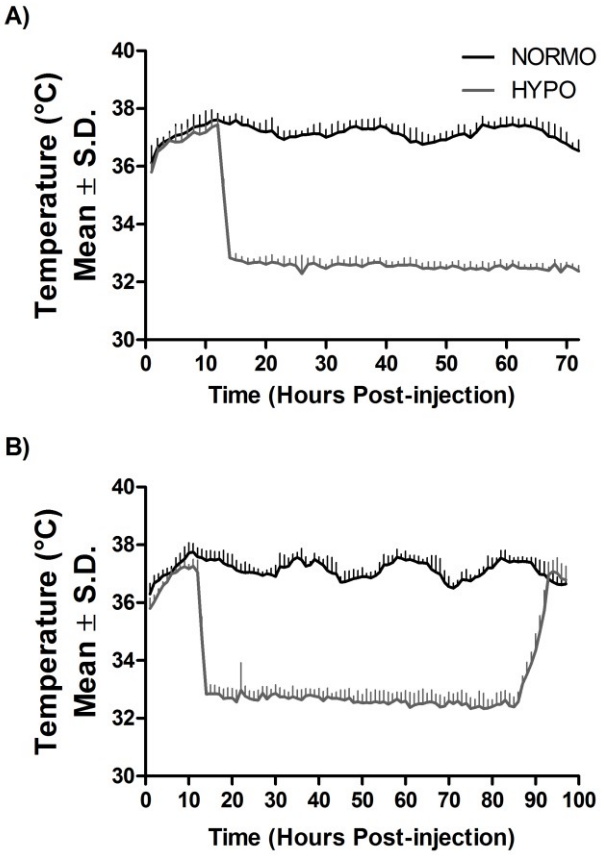

Fig. 3. Core temperature data, measured by telemetry, of a subset of animals from experiment two, which is representative of the other experiments. The temperature control system used in all experiments significantly lowered whole-body temperature beginning 12 hours after ICH and lasting 24 or 72 hours in treated animals. (A) Temperature data for animals surviving to day 3. TH-treated animals were euthanized cold. (B) Four days of temperature data for animals surviving to day 7. After 72 hours of TH, animals under-went 6 hours of rewarming (0.5°C/hour), followed by normothermia until euthanasia. n = 9 in NORMO-Day 3, n = 6 in HYPO-Day 3, n = 9 in NORMO-Day 7, and n = 6 in HYPO-Day 7.

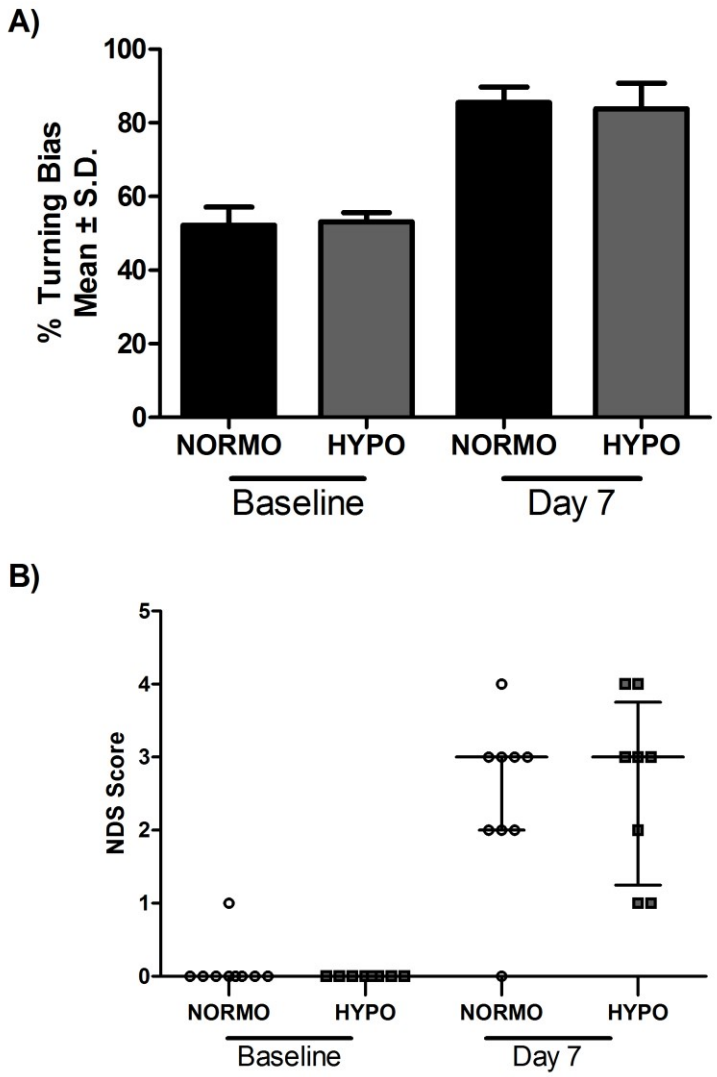

Fig. 4. Seven days after ICH, there were significant behavioral impairments on both the (A) CTT and (B) NDS (vs. baseline, $p < 0.001$), but there was no effect of TH on either test ($p \geq 0.316$). n = 9 in NORMO and n = 8 in HYPO. Raw NDS scores are plotted along with a horizontal bar representing the median score for each group with I.Q.R. bars extending vertically.

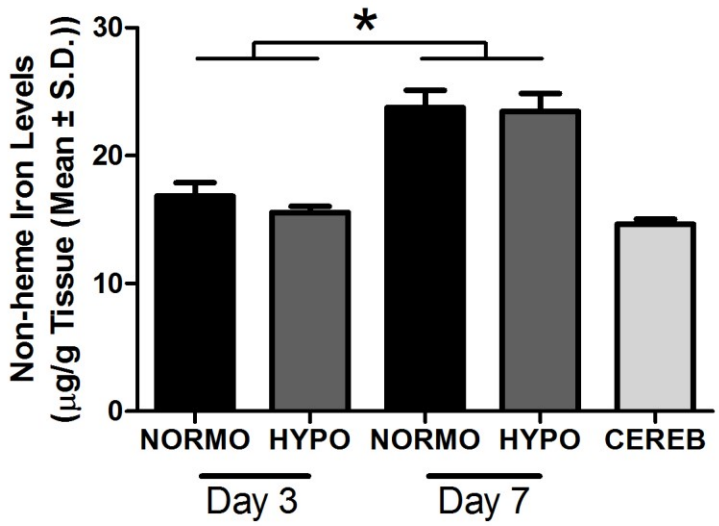

Fig. 5. There was a significant increase in non-heme iron levels in the injured forebrain from day 3 to day 7 post-ICH ($p < 0.001$) but no effect of TH ($p = 0.479$). Non-heme iron levels at day 3 were comparable to control structure levels (i.e. CEREB=cerebellum of all animals). n = 10/group. *$p < 0.05$.

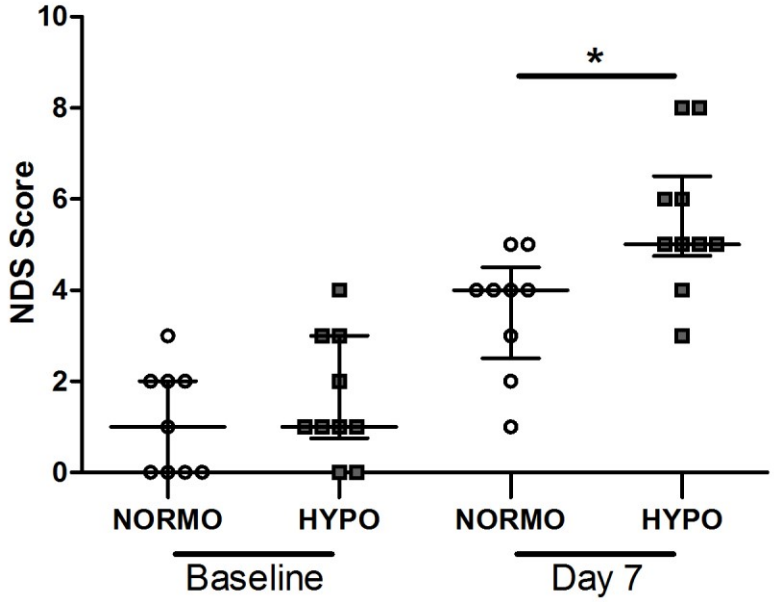

Fig. 6. There was a significant behavioral deficit as shown by NDS on day 7 post-ICH (vs. baseline, $p < 0.001$) with a slight worsening of behavioral impairments in the HYPO group ($p = 0.011$). Raw scores are plotted along with a horizontal bar representing the median score for each group. *$p < 0.05$.

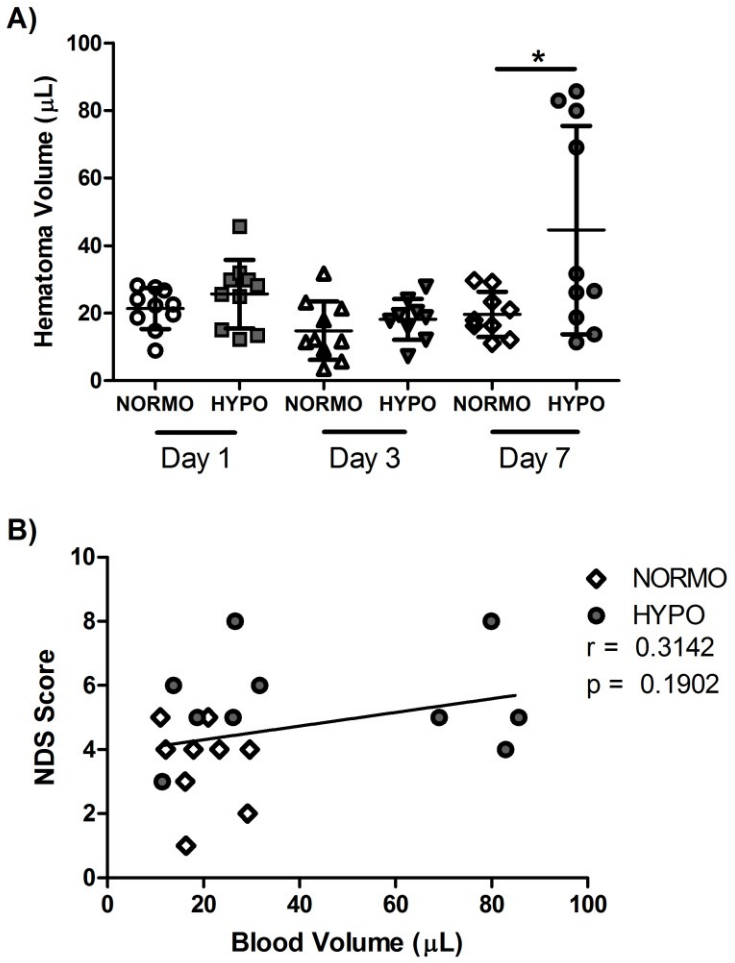

Fig. 7. (A) There was no difference between NORMO and HYPO on days 1 and 3 post-ICH ($p \geq 0.264$) but by day 7, 40% of treated animals had a significant increase in hematoma volume ($p = 0.032$). Data are expressed as injured (IPSI) hemisphere – uninjured (CONTRA) hemisphere blood volume and the raw score for each animal is plotted. The horizontal bar represents the average per group with S.D. bars extending vertically. *$p < 0.05$. (b) NDS scores did not significantly predict hematoma volume.

Chapter Five

Bipyridine, an Iron Chelator, Does Not Lesson Intracerebral Iron-Induced Damage or Improve Outcomes After Intracerebral Hemorrhagic Stroke in Rats

5.1 Introduction

Intracerebral hemorrhage (ICH) has a high mortality rate and greatly impairs survivors.[1] Currently, no clinically approved neuroprotective treatments exist. The primary damage of an ICH (i.e., mechanical injury from extravasated blood) occurs rapidly making it difficult to treat. Mechanisms causing secondary damage occur over hours to weeks and accordingly are more suitable therapeutic targets. Considerable research into secondary damage has identified possible treatments targeting inflammation, BBB disruption, edema, thrombin, and the breakdown products of erythrocytes.[2-5]

Over the initial days following an ICH, erythrocytes lyse, releasing hemoglobin into the hematoma and surrounding parenchyma.[5,6] The heme from hemoglobin is degraded causing increased levels of labile ferrous iron, which promotes oxidative stress.[5-8] This oxidative stress stems from labile iron's involvement in the Haber-Weiss reactions (i.e., the Fenton reaction) and the production of highly reactive hydroxyl radicals. As labile ferric iron catalyzes the conversion of superoxide to molecular oxygen, the ferric iron is reduced to ferrous iron. Ferrous iron catalyzes the production of hydroxyl radicals from hydrogen peroxide.[5,9,10] Reactive oxygen species, such as hydroxyl radicals, damage macro-molecules (e.g., DNA), generate other reactive species, and increase inflammation, all of which can kill neurons.[5,9,10] Indeed, a $FeCl_2$ injection into the brain causes oxidative stress,[7,11] edema,[11] epileptic activity, lesions, and an inflammatory response.[12] Furthermore, a striatal injection of $FeCl_2$ in rats causes significant, progressive lesion enlargement, neuronal degeneration, and dendritic atrophy,[13] which mirror the findings in the collagenase ICH model.[14]

Numerous animal studies have evaluated the efficacy of iron chelators[4,15,16] and deferoxamine, an intracellular and extracellular ferric iron chelator, has completed phase 1

clinical trials.[17] Deferoxamine has shown neuroprotective,[15,16] antioxidant, and anti-inflammatory effects;[4,16] however, there are other studies that find no short-term[8,18,19] or long-term neuroprotective benefit.[19] Deferoxamine binds to ferric iron and although both ferric and ferrous iron catalyze the Haber-Weiss reaction, it is ferrous iron that contributes to the formation of hydroxyl radicals. Recently, there has been interest in 2,2′-bipyridine, an intracellular ferrous iron chelator. An in vitro comparison between deferoxamine and bipyridine found that bipyridine has better cell membrane permeation and intracellular iron sequestering ability than deferoxamine.[20] Bipyridine binds the potentially more harmful ferrous iron and has superior cell permeation; therefore, it may be a more suitable treatment for ICH.

Bipyridine has been studied as a treatment for several forms of stroke. In models of subarachnoid hemorrhage, bipyridine reduced vasospasm and cell death[21] whereas in ischemia, bipyridine diminished BBB disruption.[22] A recent study of ICH found that pre-treatment with bipyridine reduced neuronal degeneration, reactive oxygen species formation, microglia reactivity, and white matter damage whereas post-treatment decreased edema, lesion volume, and behavioral impairments in mice.[8] This study used the whole blood and collagenase models of ICH to comprehensively investigate bipyridine. Another study using the whole-blood model in rats found a reduction in protein oxidation and DNA damage with bipyridine post-treatment.[7]

The current study evaluated bipyridine in three rat models of striatal injury. An ICH was produced either via injecting collagenase, a bacterial enzyme that degrades blood vessel walls causing a spontaneous bleed and progressive tissue loss,[14,23-25] or whole blood into the striatum. Also, we used a striatal injection of $FeCl_2$ to isolate iron-mediated damage.[13] First, we measured non-heme iron levels and behavioral deficits 7 days after collagenase-induced ICH using a previously reported dose and injection regimen (25 mg/kg beginning 12 hours after ICH and then

twice a day for 3 days)[7] . Second, based on a recent study, we used a smaller dose of bipyridine injected less frequently (20 mg/kg beginning 6 hours after ICH and then once every 24 hours for 2 days)[8] and measured edema 3 days after collagenase-induced ICH as this is the typical peak of edema in rodents. The whole-blood ICH model was used to determine the effect of bipyridine (20 mg/ kg beginning 6 hours after ICH and then once every 24 hours for 2 days)[8] on tissue loss, cell death, and behavioral impairments a week post-ICH. We also determined whether bipyridine caused hypothermia, a common experimental confound in stroke research, in both ICH models. Finally, we investigated the effect of bipyridine pre-treatment (25 mg/kg beginning 2 hours prior to $FeCl_2$ injection and then once every 12 hours for 3 days) on iron-induced tissue loss, neuronal degeneration, and behavioral impairments.

5.2 Methods

Animals

All procedures complied with the Canadian Council on Ani-mal Care guidelines and were approved by the University of Alberta's Animal Care and Use Committee: Biosciences. We used 90 male Sprague-Dawley rats (~11 weeks old) single-housed in polycarbonate cages (width 38 cm, length 49 cm, and height 20 cm) with wood chip bedding. Animals were kept in a humidity and temperature-controlled room on a 12-hour light/dark schedule with *ad lib* food (Rodent Diet 5001) and water. In each experiment, animals were randomly assigned (n = 10/group, except in experiment 3 with n = 15/group) to either the bipyridine group (BIP) or the saline control group (SAL). All behavioral testing and data analysis were conducted by a researcher unaware of the treatment identity.

Experiment 1

Experiment Groups

Injections of bipyridine (25 mg/kg i.p.) or an equivalent volume of saline began 12 hours after ICH followed by an injection every 12 hours for 3 days.[7] During the light cycle, rats underwent baseline behavior testing prior to ICH and behavioral testing 7 days post-ICH, after which they were euthanized for non-heme iron level analysis. Animals were acclimatized to the testing room 30 minutes prior to beginning behavioural assessment.

Collagenase ICH Surgery

Surgical procedures were performed aseptically as previously described.[14,23-25] Briefly, rats were anesthetized with isoflurane (4% induction, 2-2.5% maintenance, 60% N_2O balance O_2) and a hole was drilled at 3.5 mm right and 0.5 mm anterior to the bregma into the skull. A 26-gauge needle was inserted 6.5 mm deep from the surface of the skull and 0.7 μl of collagenase (0.14 U) was injected. The wound was closed and Marcaine was applied to diminish postoperative pain.

Neurological Deficit Scale

Neurological deficit scales (NDS) are sensitive to striatal ICH.[23,26] Rats were scored on the following tasks: spontaneous circling, hind limb retraction, bilateral forepaw grasp, contra-lateral forelimb flexion, and beam walking ability. Each task had a score range of 0-3 except contralateral forelimb flexion, which had a range of 0-2. The sum of the score ranges from 0 to 14 with 14 denoting maximum impairment.

Corner Turn Test

The Corner Turn Test (CTT) evaluates an animal's turning bias out of a corner and is sensitive to striatal injury.[19,27] The rats were placed in front of two walls (41 cm height and 30.5 cm length) that made a 30° angle and the direction of turning out of the corner was recorded. Baseline testing was completed over two consecutive days and the average performance over ten trials for each day was determined. Ten trials were done and averaged at the day 7 test time. Data for this test were excluded for rats that exhibited a turning bias (>70 or <30% for one direction) at baseline.

Ladder Test

The walking ability of the rats was assessed using a horizontal ladder, a measurement sensitive to striatal ICH.[26,28] Animals crossed the 1-m ladder made of steel rungs randomly spaced 1-4 cm apart (3-mm diameter), food and a shelter structure were used to motivate crossing. The day prior to baseline, rats underwent training (i.e., 4 crosses). Four crosses for each rat were video-recorded at baseline and four at testing on day 7. These were then analyzed for the average number of steps and slips. At any testing time, rats that crossed fewer than two times were excluded from analysis for this test.

Non-heme Iron Assay

Seven days after ICH rats were euthanized (isoflurane anesthesia and decapitation), and a 6-mm thick coronal section of both forebrains was taken from each animal (2 mm anterior to 4 mm posterior to the site of collagenase injection). Tissue taken from the ipsilateral forebrain, contralateral forebrain, and cerebellum (Cb) was homogenized.[18,29] Briefly, tissue proteins were precipitated and the samples were centrifuged. The supernatant was collected and reacted with a

ferrozine chromogen solution for a colorimetric assessment of the tissue's non-heme iron concentration.

Experiment 2

Experiment Groups

Rats were given bipyridine (20 mg/kg i.p.) or saline 6 hours after ICH and then once every 24 hours until euthanasia. This change in dose regimen was due to a recent publication on bipyridine after ICH.[8] Most of the rats (BIP n = 8, SAL n = 7) underwent a core probe implantation to measure body temperature. Rats were euthanized 72 hours after ICH for edema measurement.

Core Temperature Probe Implantation

Four days before ICH, rats were implanted with core temperature telemetry probes (TAT10TA-F20 or F40, Transoma Medical, St. Paul, MN, USA). Briefly, animals were anesthetized with isoflurane and the probe was surgically implanted into the peritoneal space.[30] Marcaine was applied to the wound and the animals recovered in a clean cage. Temperature data, sampled every 30 seconds, were taken beginning 24 hours prior to ICH surgery (baseline) and until euthanasia.

ICH Surgery

Surgical procedures were the same as in experiment 1.

Brain Water Content

The rats were euthanized 72 hours post-ICH (decapitation under isoflurane) to measure brain water content (BWC).[19,30] A 6-mm coronal section of the ipsilateral and contralateral fore-brains was taken (2 mm anterior to 4 mm posterior the injection site) along with the Cb as a control. The cortex and striatum were separated in each forebrain for individual weight measurement before and after being baked at 100°C for 24 hours. In computing for BWC, we used the following formula:

%BWC = [(wet weight−dry weight) / wet weight] × 100.

Experiment 3

Experimental Groups

The same bipyridine treatment used in experiment 2 was used in this experiment. We chose this treatment as opposed to the greater dosage in experiment 1 since there was no discoloration of rat feces in experiment 2 (i.e., side effects). Rats were subjected to a whole-blood ICH and tested for behavioral deficits followed by euthanasia 7 days post-insult. Most rats underwent core temperature probe implantation 4 days prior to ICH (n = 7/group).

Whole-Blood ICH Surgery and Core Probe Implantation

Surgery was the same as in experiment 1 except that instead of collagenase, 100 μL of whole blood (from tail vein) was infused into the striatum over 10 min and the needle was left in place for an additional 10 min.[24,30] The same surgical procedures for probe implantation used in experiment 2 were used here.

Behavioral Testing

The same behavioral testing was done as in experiment 1.

Lesion Volume

The rats were euthanized with pentobarbital (100 mg/kg i.p.) and transcardially perfused with 0.9% saline followed by 10% formalin. The brain sections (40 μm) were stained with cresyl violet. One in every ten sections was analyzed for lesion analysis using ImageJ[30] as follows:

Volume of a hemisphere = average (area of the complete coronal section of the hemisphere – area of ventricle – area of damage) × interval between sections × number of sections

Tissue lost = remaining volume of normal hemisphere – remaining volume of injured hemisphere.

Fluoro-Jade Stain

Degenerating neurons were identified using Fluoro-Jade B.[13,31] The slides were prepared and incubated with 0.001% Fluoro-Jade (Chemicon, Temecula, CA, USA). Fluoro-Jade positive cells (FJ+) were visualized with a fluorescein filter, and the total number of cells for one section per rat was counted at the level of the maximum hematoma size.

Experiment 4

Experiment Groups

This experiment tested a pre-treatment of bipyridine on iron-induced damage. Two hours before $FeCl_2$ injection, rats received bipyridine (25 mg/kg i.p.) or saline and then an injection every 12 hours for 3 days. Animals underwent behavioral testing 7 days after $FeCl_2$ injection and were euthanized to determine lesion volume and neurodegeneration.

$FeCl_2$ Surgery

Surgery was the same as experiment 1 except we infused 3.8 μg of $FeCl_2$ in a 30-μl solution of sterile unbuffered saline over 10 min.[7,13] This solution was mixed and kept frozen until used.

Behavioral Testing

The same three tests used in experiment 1 were used here.

Lesion Volume and Fluoro-Jade Stain

The same procedures used in experiment 3 were used here.

Experiment 5

A spectrometry assay was performed to confirm that our preparation of bipyridine chelated iron at a 3:1 mole ratio.[32] The concentration of $FeCl_2$ was kept constant (12.68 μg $FeCl_2$ in 10 ml of saline) and mixed with increasing concentrations of bipyridine (10 mM of bipyridine was prepared which contained 97.6125 mg of bipyridine in 62.5 ml of saline and then diluted to 1, 2, 3, 4, 5, 6, and 7 mM). The readings were measured at a wavelength of 556 nm. Saline+bipyridine, saline+$FeCl_2$, and saline alone were used as controls.

Statistical Analysis

The data are presented as mean ± standard deviation (SD), except for NDS scores that are presented as medians. Data was analyzed via repeated measures and/or one-way ANOVA using SPSS, except the NDS scores that were analyzed by Mann-Whitney and Wilcoxon tests (SPSS v.17.0, SPSS Inc, Chicago, IL, USA).

5.3 Results

There was no mortality in any experiment. One rat was excluded from the SAL group in experiment 3 due to surgical error.

Experiment 1

The percent drop in weight (from surgery) in the BIP group ranged from 10.7 to 11.6% on days 1-3 post-surgery, while the SAL group lost 2.7% on day 1 and gained 2.1% by day 3.

There was a significantly greater loss in the BIP group on all 3 days ($p \leq 0.001$). Animals treated with bipyridine had orange discoloration of their feces.

Behavioral Outcome

Three rats were excluded from CTT due to a baseline turning bias (two from BIP and one from SAL). There were no exclusions in NDS and the ladder test. Baseline NDS scores were normal and there was no difference between groups ($p = 0.549$, Fig. 1(A)). There was significant impairment 7 days post-ICH ($p < 0.0001$ vs. baseline); however, there was no difference between groups ($p = 0.646$). For the CTT, there was a significant time effect ($p < 0.0001$, Fig. 1(B)) but no group effect ($p = 0.718$) or interaction ($p = 0.222$). In ladder test, there was a significant time effect ($p < 0.0001$, Fig. 1(C)) but no group effect ($p = 0.259$) or interaction ($p = 0.538$).

Non-heme Iron Levels

There was a significant increase in non-heme iron levels (μg iron/g of brain tissue) in the injured forebrain (BIP 35.43 ± 18.45, SAL 30.40 ± 4.49) compared to the non-injured forebrain (BIP 13.87 ± 1.93, SAL 14.65 ± 2.9, $p < 0.0001$) and the cerebellum (BIP 14.08 ± 1.78, SAL

15.64 ± 3.05, $p \leq 0.002$) but no difference between groups ($p = 0.413$). Thus, bipyridine did not alter iron levels after ICH.

Experiment 2

The body weight dropped in the BIP group (1.8–4.5%) over the first 3 days post-ICH whereas for the SAL group, there was a 1.7% drop on the first day but a 2.3% increase by the third day. The group differences in weight change were significant ($p \leq 0.002$).

BWC

Edema was measured 3 days post-ICH (Fig. 2). There was a significant increase in BWC in the injured striatum ($p < 0.0001$) and cortex ($p = 0.009$) compared to the uninjured side and the Cb ($p < 0.0001$). Bipyridine did not reduce edema in the ipsilateral cortex or striatum ($p \geq 0.593$).

Body Temperature

Bipyridine transiently reduced temperature (Fig. 3(A)), but there was no difference between groups at baseline ($p = 0.828$). The first injection 6 hours after ICH caused the greatest cooling ($p \leq 0.029$) that lasted for 5 hours. The second and third injections also caused significant but shorter hypothermia ($p \leq 0.037$ vs. SAL on hourly averaged data).

Experiment 3

Although the whole-blood model caused weight loss over the first 3 days, bipyridine did not worsen it ($p = 0.922$).

Behavioral Outcome

One SAL rat was excluded from the CTT analysis due to a baseline turning bias in this test. In all behavioral tests, ICH caused significant impairment ($p < 0.0001$ vs. baseline). There was no difference between groups in NDS ($p = 0.804$, Fig. 4(A)). In CTT there was no group effect ($p = 0.683$, Fig. 4(B)) or interaction ($p = 0.336$), and again for ladder there was no group effect ($p = 0.318$) or interaction ($p = 0.860$, Fig. 4(C)).

Lesion Volume and Neurodegeneration

Bipyridine did not lessen tissue loss ($p = 0.097$, Fig. 5(A)) or the number of FJ+ cells ($p = 0.790$ Fig. 5(B)) a week post-hemorrhage.

Body Temperature

Bipyridine caused transient cooling after each injection ($p \leq 0.001$, Fig. 3(B)), but there was no difference between groups at baseline ($p = 0.871$).

Experiment 4

The BIP group lost greater weight ($p \leq 0.011$) on all 3 days post-$FeCl_2$ injection, while the SAL group gained weight (drop of 3.5% vs. gain of 1.8% at day 3).

Behavioral Outcome

Three rats in each group were excluded from the CTT due to a turning bias, and one rat from the SAL group was excluded from the ladder test because of insufficient crossing. Baseline NDS scores were normal. At 7 days after $FeCl_2$ injection, there was significant impairment ($p < 0.0001$ vs. baseline, Fig. 6(A)) but no difference between groups ($p = 0.098$). Results for CTT showed a time effect ($p = 0.001$) but no group effect ($p = 0.879$) or interaction ($p = 0.802$, Fig. 6(B)). In the ladder test, there was a significant time effect ($p < 0.0001$) but no group effect ($p =$

0.243) or interaction (p = 0.238, Fig. 6(C)). Thus, a striatal injection of $FeCl_2$ caused impairment, which bipyridine did not attenuate.

Lesion Volume and Neurodegeneration

Bipyridine did not ameliorate tissue loss (p = 0.332, Fig. 5(C)) or the number FJ+ cells (p = 0.657, Fig. 5(D)) at a week post-insult.

Experiment 5

Bipyridine chelated increasing concentrations of $FeCl_2$ until saturation at a ratio of 3 moles of bipyridine per mole of iron (data not shown). At least in a test tube, bipyridine was chemically active as expected.

5.4 Discussion

Despite using three models, two drug doses, pre- and post-injury regimens, and multiple endpoints, we failed to find any effect of bipyridine. Specifically, post-treatment did not impact parenchymal non-heme iron levels, behavioral impairments, or edema after a collagenase-induced ICH. Nor did bipyridine lessen tissue loss, cell death, or behavioral impairment in the whole-blood ICH model. Finally, pre- and post-treatment did not influence the $FeCl_2$-induced lesion, neurodegeneration, or behavioral impairment. Thus, these results are contrary to the considerable literature supporting the use of iron chelators for ICH.[7,8,15,17]

Our study has several general limitations. First, in any negative study, it remains possible that other drug doses or treatment regimens could provide neuroprotection. Thus, we cannot exclude this possibility even after conducting four efficacy experiments using two different drug doses and treatment regimens. Second, despite considerable methodological overlap with

previous studies (e.g., dose regimens, models, and endpoints),[7,8] some factors or combinations of factors may differ among studies and be critically important to drug-induced neuroprotection. Unfortunately, the present findings do not explain why others find a neuroprotective effect whereas we do not. Similarly, our lab's failure to obtain benefit with deferoxamine[18,19] stands in contrast to a considerable amount of work supporting its neuroprotective effects. Again, there was an overlap in drug dosage, endpoints, etc. Third, small treatment effects may have gone unnoticed in our studies despite using above-average group sizes and multiple endpoints that are widely used and well established in the field.[33] Notably, there were no trends suggesting the benefit in our study. Fourth, we did not prove that bipyridine entered and chelated iron in the brain. There is no simple way to do this, and while we could have assessed whether bipyridine affected oxidative stress, we did not, owing to the lack of neuroprotective and behavioral effect. However, we did show that bipyridine, mixed fresh prior to each injection, was chemically active in a dish. As well it was clear the drug affected the animals (i.e., discolored feces, weight loss, and hypothermia). Of note in experiment 4, some animals pre-treated with bipyridine displayed significant movement abnormalities (e.g., high-amplitude shaking including aberrant tremulous movement of head, trunk, and limbs) once they awoke from anesthesia, which we have never observed previously including in rats given striatal ICH or $FeCl_2$ infusion. This behavior subsided within an hour but suggests that bipyridine does reach the striatum and thus interacts with the $FeCl_2$. Besides the general limitations common to most negative studies, each experiment here has its own limitations.

The first study confirmed the increase in non-heme iron levels beginning a few days after ICH.[6,18] Bipyridine did not lower iron levels (i.e., improve iron clearance), but it is possible the non-heme assay is not sensitive enough to detect a treatment effect, which may have been

observed with other methods such as X-ray fluorescence imaging.[18] Also, while we did not assess cell death in this model, the behavioral data, as well as the histology and behavior of our other experiments, suggest (but do not prove) that there would be no neuroprotection in the collagenase model either.

The second study showed that bipyridine did not reduce edema 3 days after collagenase-induced ICH. Possibly, edema may have been affected at other times but this time point was chosen as it corresponds to the peak edema time in rats and is commonly used. As well, Wu and others[8] found bipyridine to reduce edema in the whole-blood model in mice. Thus, species or model differences likely account for study discrepancies. It is also possible that the bipyridine-induced hypothermia contributed to the reduction in edema found in mice, especially if the cooling was more pronounced or occurred at the time of euthanasia in that study. Hypothermia reduces edema after ICH without consistently conferring neuroprotection or improving behavioral impairments.[30] Note that at euthanasia, our animals were no longer colder than the controls (Fig. 3(A)).

As we only assessed the histological and behavioral effect of bipyridine in the third and fourth experiments, some benefit may have gone unmeasured. However, owing to the lack of histological and behavioral effect, as well as the null results on edema and iron levels in the collagenase model, we did not see a reason to pursue other endpoints (e.g., oxidative stress).

Bipyridine failed to attenuate iron-induced neuronal damage and behavioral impairment in experiment 4 despite pre-and post-treatment. An injection of $FeCl_2$ is used as a simplified model to mimic ICH and traumatic injury, but there are limitations. For instance, a single bolus dose in an acidic solution (pH 5) does not mimic the slow release of iron from degrading

erythrocytes at physiological pH. However, this model has been previously used in mechanistic studies of ICH[7,13] and we have shown that it is the iron not the acidity that causes injury in this model.[13] While pH may influence bipyridine's chelating abilities, this is likely a transient effect as the body buffers the solution. Still, this model has limited face validity and although we attempted to study whether a striatal injection of bipyridine and $FeCl_2$ solution would cause injury, rats displayed similar behavioral abnormalities as described and we terminated the study owing to health concerns. Finally, while this model resulted in similar behavioral deficits produced by a whole-blood injection, $FeCl_2$ caused a slightly milder turning bias and somewhat worse walking impairment compared to collagenase. This may relate to subtle differences in lesion size and placement or to other factors (edema, plasticity responses, etc.). However, as we have previously shown, even matching the whole blood and collagenase models is exceedingly difficult, as each model has markedly different outcomes despite matching of the initial hematoma volumes.[24]

There are several reasons to explain why bipyridine did not prevent the iron-induced damage. Conditions within the brain after injury (e.g., pH changes) may impede the chelator's ability to bind iron. It is also possible that bipyridine did not reach the injured striatum. Yet, we used similar treatment protocols that others have reported benefit with, and the abnormal behavior immediately post-surgery suggests that bipyridine does reach the striatum. Alternatively, injections lasting only 3 days after injury may not be sufficient to provide neuroprotection and improve behavioral recovery. Okauchi and others[15] found that only prolonged periods of deferoxamine treatment (7-14 days) provided lasting histological and behavioral benefit. However, a longer duration of deferoxamine treatment (i.e., 7 days) has not always provided protection.[19] Finally, it is also possible that chelators bind only to a portion of

the labile iron pool and there is enough left unbound to generate potent hydroxyl radicals. Hydroxyl radicals, along with damaging macromolecules, generate a cascade of reactive species leading to a snowballing effect of oxidative damage.[9] Of note, a possible adverse effect of iron chelators is that they may interfere with the brain's endogenous response to increased non-heme iron levels (i.e., upregulating ferritin) thereby making the brain vulnerable to un-chelated iron.[34]

Iron from degrading erythrocytes poses a significant risk to surrounding tissue[2,5-7,13,16] and thus is a potential mechanism of injury to target. Animal studies show a persistent increase in non-heme iron levels from 3 to 7 days after ICH and these levels remain elevated up to 28 days.[6,18] In patients, magnetic resonance imaging suggests that iron deposits expand out from the initial hematoma into the surrounding parenchyma over 3 months.[35] As iron from an ICH is not readily cleared, the brain is vulnerable to iron-induced oxidative injury. Even protein-bound iron (e.g., ferritin) may be liberated by free radicals (e.g., superoxide) thereby propagating formation of more reactive species.[9] Although iron causes secondary cell death, there are several other secondary mechanisms of injury after ICH. Thus, while bipyridine may chelate ferrous iron, this may not be enough to improve the overall outcome. Combination treatments or those with pleiotropic effects are more likely to provide neuroprotection. For example, antioxidants (e.g., NXY-059) have failed clinical trials[36] possibly because these drugs (e.g., ascorbate in the presence of molecular oxygen), when catalyzed by insufficiently bound iron, can become pro-oxidants.[9] Therefore, it has been suggested that combinations of iron chelators with antioxidants will provide better protection than either alone.[9] Sulforaphane, an isothiocyanate, promotes the activity of nuclear factor E2-factor related factor, a transcription factor that acts on the antioxidant response element[37] thereby increasing expression of several different antioxidants[37,38] and attenuating behavioral impairments, inflammation, and oxidative stress

after ICH.[38] Sulforaphane also promotes expression of haptoglobin, a hemoglobin-binding protein, thereby preventing release of iron.[39]

In summary, our results do not support the use of bipyridine as a single treatment approach for ICH. This study comprehensively evaluated bipyridine using several models and found the drug ineffective for all endpoints. Thus, iron chelators do not accomplish enough on their own to provide neuroprotection and improve behavioral impairments. Many have argued for the publication of negative animal data in order to provide a more thorough picture prior to clinical research.[40] In this regard, our results suggest that further experimentation is needed on iron chelators before further movement through clinical ICH studies.

5.5 References

1. Sacco S, Marini C, Toni D, Olivieri L, Carolei A. Incidence and 10-year survival of intracerebral hemorrhage in a population-based registry. Stroke 2009 Feb 1,;40(2):394-399.

2. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

3. Rincon F, Mayer SA. Intracerebral hemorrhage: Clinical overview and pathophysiologic concepts. Translational stroke research 2012 Jul;3(Suppl 1):10-24.

4. Selim M. Deferoxamine mesylate: A new hope for intracerebral hemorrhage: From bench to clinical trials. Stroke 2009 Mar 1,;40(3, Supplement 1):S90-S91.

5. Wagner KR, Sharp FR, Ardizzone TD, Lu A, Clark JF. Heme and iron metabolism: Role in cerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2003 Jun;23(6):629-652.

6. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke 2003 Dec 1,;34(12):2964-2969.

7. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

8. Wu H, Wu T, Li M, Wang J. Efficacy of the lipid-soluble iron chelator 2,2'-dipyridyl against hemorrhagic brain injury. Neurobiol Dis 2012 Jan;45(1):388-394.

9. Kell DB. Iron behaving badly: Inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC Med Genomics 2009 Jan 08,;2:2.

10. Kell DB. Towards a unifying, systems biology understanding of large-scale cellular death and destruction caused by poorly liganded iron: Parkinson's, huntington's, alzheimer's, prions, bactericides, chemical toxicology and others as examples. Arch Toxicol 2010 Nov;84(11):825-889.

11. Willmore LJ, Rubin JJ. Formation of malonaldehyde and focal brain edema induced by subpial injection of FeCl2 into rat isocortex. Brain research 1982 Aug 19,;246(1):113-119.

12. Willmore LJ, Ballinger WE, Boggs W, Sypert GW, Rubin JJ. Dendritic alterations in rat isocortex within an iron-induced chronic epileptic focus. Neurosurgery 1980 Aug;7(2):142-146.

13. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

14. Nguyen AP, Huynh HD, Sjovold SB, Colbourne F. Progressive brain damage and alterations in dendritic arborization after collagenase-induced intracerebral hemorrhage in rats. Current neurovascular research 2008 Aug;5(3):171.

15. Okauchi M, Hua Y, Keep RF, Morgenstern LB, Schallert T, Xi G. Deferoxamine treatment for intracerebral hemorrhage in aged rats: Therapeutic time window and optimal duration. Stroke 2010 Feb 1,;41(2):375-382.

16. Wu H, Wu T, Xu X, Wang J, Wang J. Iron toxicity in mice with collagenase-induced intracerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2011 May;31(5):1243-1250.

17. Selim M, Yeatts S, Goldstein JN, Gomes J, Greenberg S, Morgenstern LB, et al. Safety and tolerability of deferoxamine mesylate in patients with acute intracerebral hemorrhage. Stroke 2011 Nov;42(11):3067-3074.

18. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

19. Warkentin LM, Auriat AM, Wowk S, Colbourne F. Failure of deferoxamine, an iron chelator, to improve outcome after collagenase-induced intracerebral hemorrhage in rats. Brain Research 2009;1309:95-103.

20. Kress GJ, Dineley KE, Reynolds IJ. The relationship between intracellular free iron and cell injury in cultured neurons, astrocytes, and oligodendrocytes. Journal of Neuroscience 2002 Jul 15,;22(14):5848-5855.

21. Yu YY, Niu L, Gao L, Zhang GL, Li J, Deng JP, et al. Ferrous chelator 2,2-dipyridyl attenuates cerebral vasospasm after experimental subarachnoid haemorrhage in rabbits. The Journal of International Medical Research 2010 Mar;38(2):583-592.

22. Méthy D, Bertrand N, Prigent-Tessier A, Mossiat C, Stanimirovic D, Beley A, et al. Beneficial effect of dipyridyl, a liposoluble iron chelator against focal cerebral ischemia: In vivo

and in vitro evidence of protection of cerebral endothelial cells. Brain Research 2007;1193:136-142.

23. Del Bigio MR, Yan HJ, Buist R, Peeling J. Experimental intracerebral hemorrhage in rats. magnetic resonance imaging and histopathological correlates. Stroke 1996 Dec;27(12):2312-2320.

24. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

25. Rosenberg GA, Mun-Bryce S, Wesley M, Kornfeld M. Collagenase-induced intracerebral hemorrhage in rats. Stroke 1990 May;21(5):801-807.

26. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

27. Schallert T. Behavioral tests for preclinical intervention assessment. NeuroRx 2006 Oct;3(4):497-504.

28. Metz GA, Whishaw IQ. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: A new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 2002 Apr;115(2):169-179.

29. Rebouche CJ, Wilcox CL, Widness JA. Microanalysis of non-heme iron in animal tissues. J Biochem Biophys Methods 2004 Mar 31;58(3):239-251.

30. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

31. Schmued LC, Hopkins KJ. Fluoro-jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 2000 Aug;874(2):123-130.

32. Pedersen BK. The diseasome of physical inactivity--and the role of myokines in muscle--fat cross talk. J Physiol (Lond) 2009 Dec 01,;587(Pt 23):5559-5568.

33. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

34. Pedersen BK, Pedersen M, Krabbe KS, Bruunsgaard H, Matthews VB, Febbraio MA. Role of exercise-induced brain-derived neurotrophic factor production in the regulation of energy homeostasis in mammals. Exp Physiol 2009 Dec;94(12):1153-1160.

35. Wu G, Xi G, Hua Y, Sagher O. T2* magnetic resonance imaging sequences reflect brain tissue iron deposition following intracerebral hemorrhage. Transl Stroke Res 2010 Mar 01,;1(1):31-34.

36. Lyden PD, Shuaib A, Lees KR, Davalos A, Davis SM, Diener H, et al. Safety and tolerability of NXY-059 for acute intracerebral hemorrhage: The CHANT trial. Stroke 2007 Aug 1,;38(8):2262-2269.

37. Fitch CA, Song Y, Levenson CW. Developmental regulation of hepatic ceruloplasmin mRNA and serum activity by exogenous thyroxine and dexamethasone. Proc Soc Exp Biol Med 1999 May;221(1):27-31.

38. Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, et al. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. Stroke 2007 Dec;38(12):3280-3286.

39. Zhao X, Song S, Sun G, Strong R, Zhang J, Grotta JC, et al. Neuroprotective role of haptoglobin after intracerebral hemorrhage. J Neurosci 2009 Dec 16,;29(50):15819-15827.

40. Lapchak PA, Zhang JH. Resolving the negative data publication dilemma in translational stroke research. Transl Stroke Res 2011 Mar;2(1):1-6.

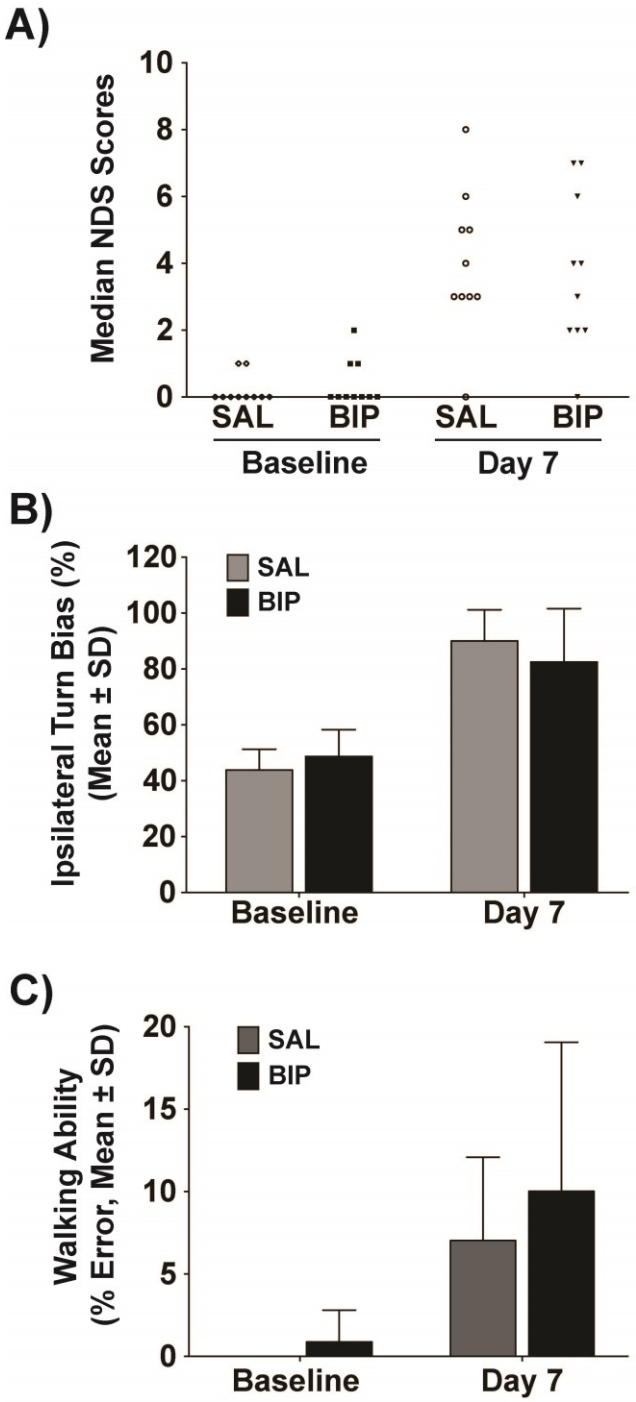

Fig. 1. The collagenase-induced ICH caused significant impairments on (A) NDS, (B) CTT, and (C) ladder tests (vs. baseline, $p < 0.0001$), but there was no significant effect of bipyridine treatment ($p \geq 0.259$). NDS and Ladder: n = 10/group; CTT: n = 9 in SAL, and n = 8 in BIP.

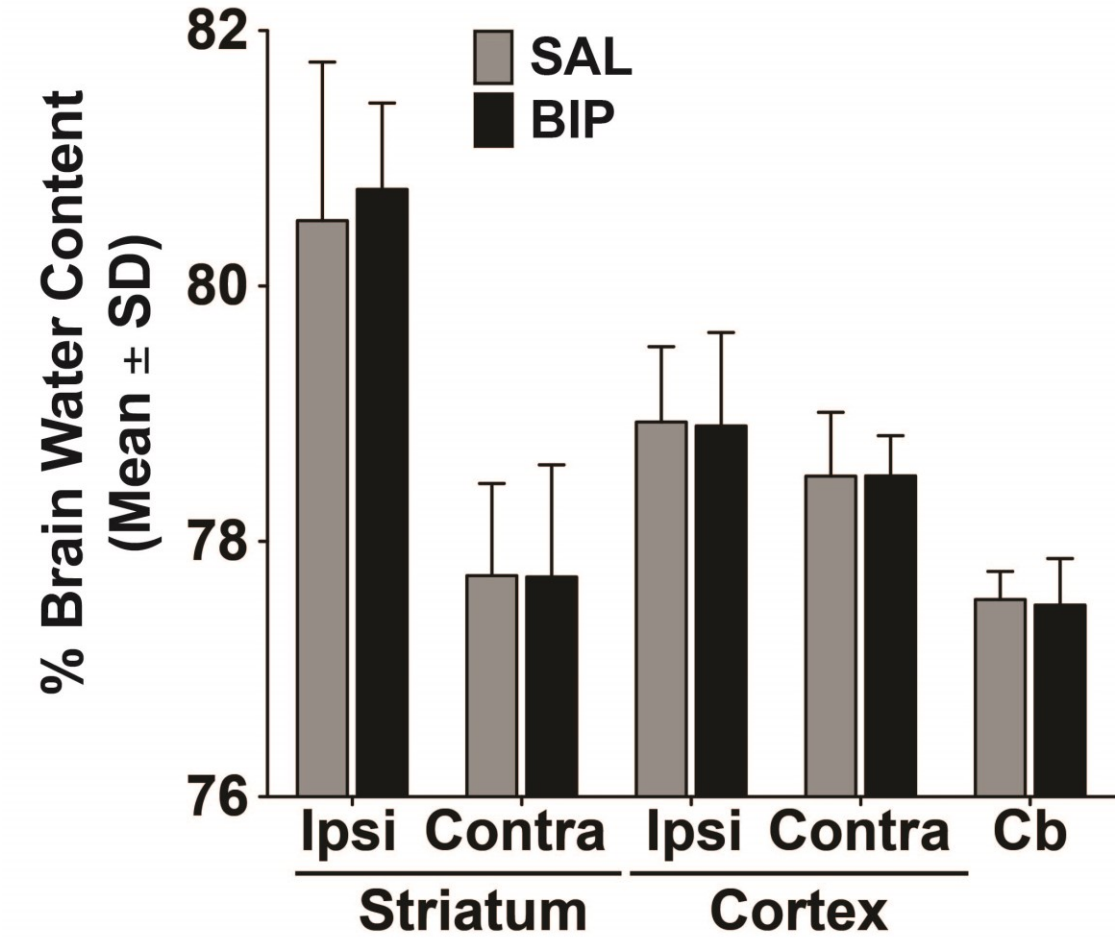

Fig. 2. Collagenase-induced ICH significantly elevated striatal and cortical BWC at 3 days (vs. contralateral side and cerebellum (Cb), $p \leq 0.009$). Bipyridine had no significant effects ($p \geq 0.593$). n = 10/group.

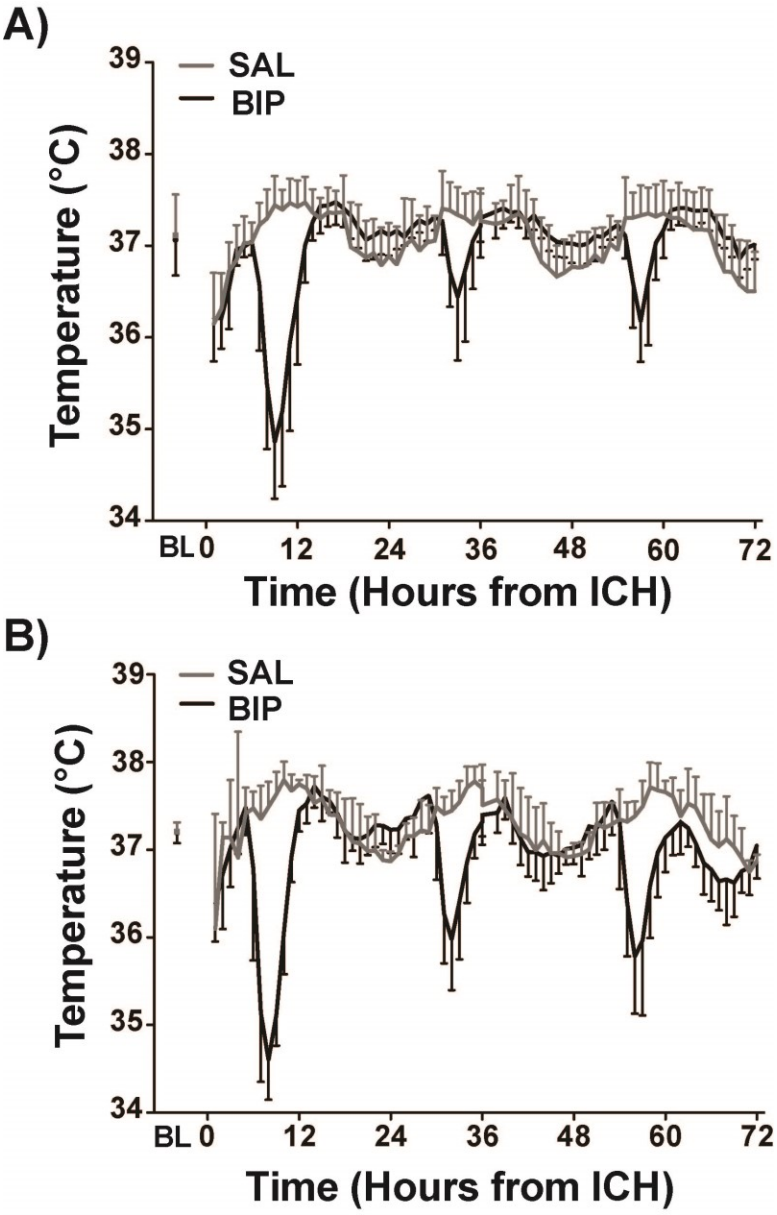

Fig. 3. Body temperature (°C) was measured using telemetry probes before and after (A) collagenase (n = 7 in SAL, n = 8 in BIP) and (B) blood infusions (n = 7/group). There was no difference in baseline temperature but there was significant, transient cooling after each bipyridine injection (6, 30, and 54 hours after ICH, (A) $p \leq 0.029$, (B) $p \leq 0.001$).

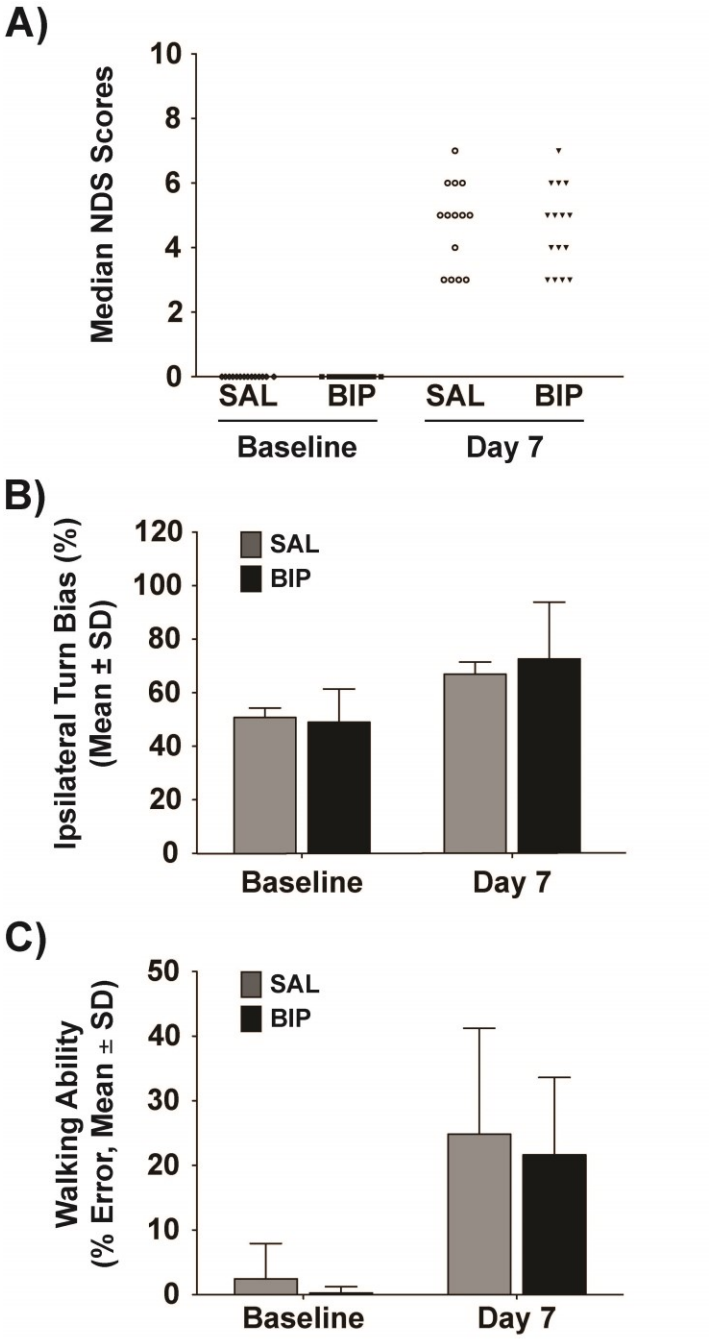

Fig. 4. The whole-blood infusion caused significant impairments on (A) NDS, (B) CTT, and (C) ladder tests ($p \leq 0.0001$), which bipyridine did not treat ($p \geq 0.318$). NDS and ladder: n = 14 in SAL, n = 15 in BIP; CTT: n = 13 in SAL, n = 15 in BIP.

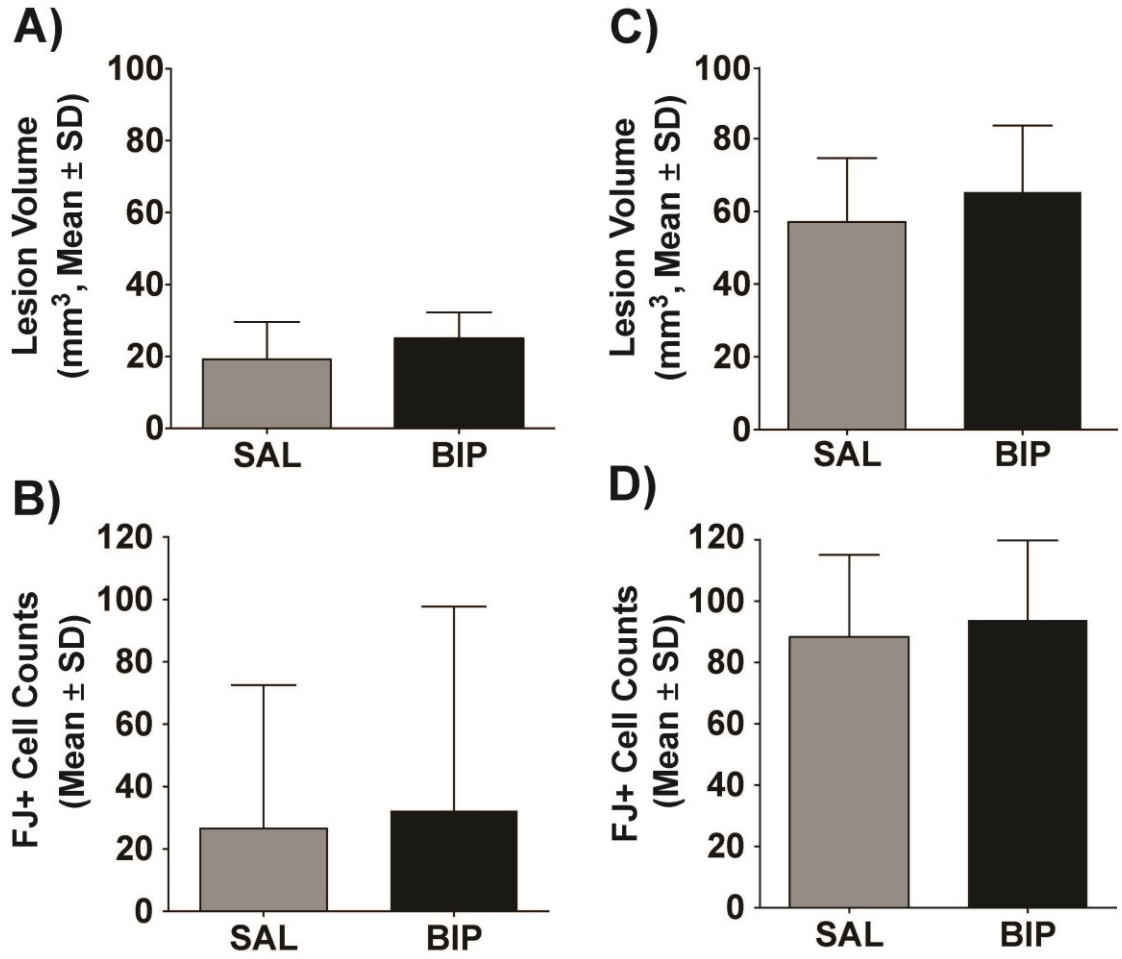

Fig. 5. Lesion volume and neuronal degeneration was assessed 7 days after blood (A, B, n = 14 in BIP, n = 15 in BIP) and $FeCl_2$ striatal infusion (C, D, n = 10/group). Lesion volume (A, C) and number of Flouro-Jade positive (FJ+) cells (B, D) were similar between groups ($p \geq 0.097$).

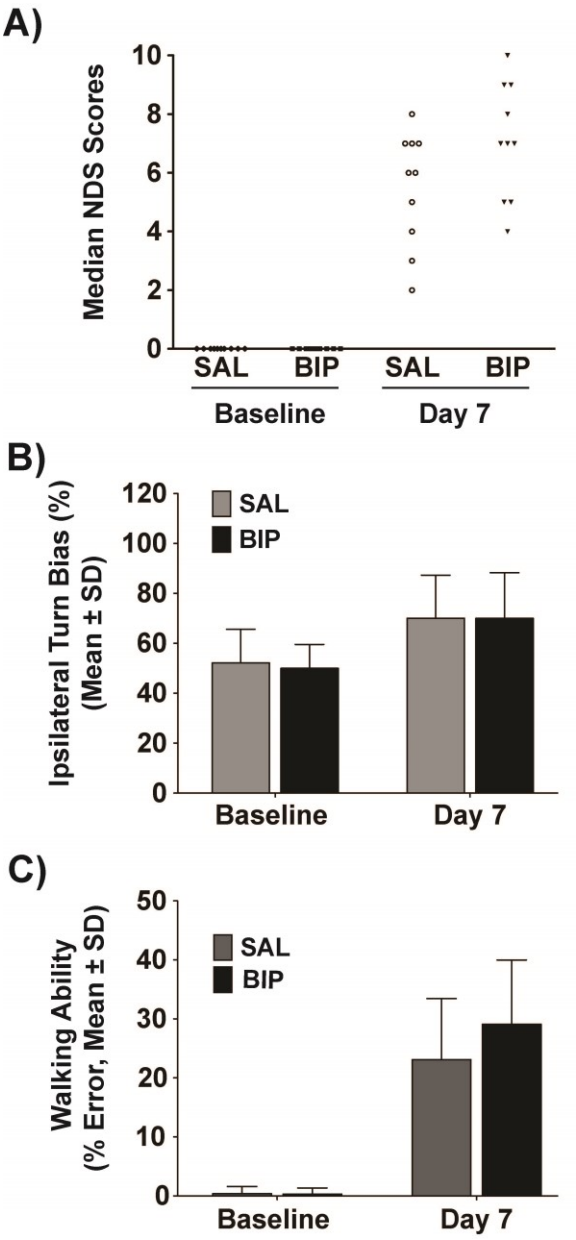

Fig. 6. A striatal $FeCl_2$ infusion caused significant deficits on (A) NDS, (B) CTT, and (C) ladder tests ($p \leq 0.0001$), which were not influenced by bipyridine ($p \geq 0.098$). NDS: n = 10/group; CTT: n = 7/group; Ladder: n = 9 in SAL, n = 10 in BIP.

Chapter Six

General Discussion

6.1 General Conclusions

The purpose of this thesis was to better understand the impact of therapeutic hypothermia (TH) on mechanisms of injury that are important for secondary injury in intracerebral hemorrhage (ICH). The literature on the effect of cooling for ICH is not as robust, especially when compared to other types of brain injury such as ischemic stroke.[1,2] We focused on the effect of TH on thrombin- and iron-induced injury, as these cause more damage following a hemorrhagic stroke than ischemic stroke. Also, we studied the influence of TH on hematoma containment and resolution. Our hope was to identify potential targets for a combination therapy where TH would be coupled with a pharmacological intervention.

Our results confirmed our hypothesis that TH does not mitigate all secondary injury that stems from an ICH; moreover, we found some unexpected but important results. Cooling does not appear to be an effective therapy against thrombin or iron injury.[3,4] Our first set of experiments show no effects of TH on brain water content or functional recovery following thrombin infusion and our data suggests TH has a transient effect on degenerating neurons. As well, while TH reduced bleeding following iron infusion, this did not lead to functional or neuroprotective effects. Our most surprising finding was that TH appears to cause an increase in blood volume following rewarming but only in a subset of animals. The inconsistency of this rebleeding would make it difficult to detect in other studies but could impact the ability to find neuroprotection in preclinical studies, especially in studies with end times after the hematoma has resolved. From these findings we concluded that TH is not effective at targeting key mechanisms of injury after ICH and can cause significant complications (i.e., rebleeding) in some subjects. In the following sections I will summarize my findings, discuss limitations and provide future directions.

6.2 Effect of Therapeutic Hypothermia on Secondary Mechanisms of Injury

Following an ICH there is on-going damage after the initial bleed. Several of the mechanisms causing this secondary injury overlap with ischemic stroke (e.g., oxidative stress, BBB disruption, etc.); however, certain mechanisms of secondary injury, such as thrombin and iron, seemingly play a large role in on-going damage after a hemorrhage compared to an ischemic stroke due to the influx of erythrocytes in hemorrhagic stroke. Apart from the hemorrhagic transformation, there is little to no generation of thrombin after ischemic stroke. And while thrombin is an essential component of the clotting cascade, which is vital for limiting hematoma expansion, it is also neurotoxic.[5-8] Iron is another mechanism of injury thought to cause proportionally more damage in a hemorrhagic stroke due the substantially greater number of erythrocytes in the brain parenchyma. While endogenous mechanisms exist, such as ferritin and superoxide dismutase, to protect against iron-induced damage, it is thought that the amount of iron released from degrading erythrocytes overwhelms those innate protective measures.[6,9,10] Both thrombin and iron are well known to be neurotoxic and to play a role in injury following ICH,[5,6,11] which makes them attractive therapeutic targets. Therefore, chapters two and three of this thesis sought to evaluate the protection of TH against these two mechanisms of injury and are summarized and discussed below.

6.2.1 Thrombin

The results from Chapter 2 suggest that TH has little to no effect on thrombin-induced injury. We did find that treated animals had significantly more degenerating neurons than the

normothermic group two weeks after insult. A previous study has shown that an infusion of thrombin does not cause on-going tissue loss past 7 days.[5] As there is no chronic cell death and we did not find an effect on either lesion volume or behavioural impairments, we inferred that TH gave some neuroprotection in our study but that this protection was transient. There was also no effect on thrombin-induced edema regardless of whether focal or systemic hypothermia was applied. Overall, our data suggests that TH does not adequately mitigate thrombin-induced injury.[3]

Our results are contradictory to others who have found cooling to mediate thrombin-induced edema.[12,13] Both groups also evaluated other mechanisms of injury (e.g., BBB permeability, matrix metalloproteinase-9 expression, etc.), but neither looked at the effects of TH on behavioural function and/or neuroprotection. While they found TH to improve several mechanisms that contribute to edema, this would not necessarily equate to functional benefit or reduced tissue loss. Edema is a commonly used endpoint in ICH animal studies but a reduction in edema does not automatically translate to neuroprotection.[14] Kawai and others[12] used an initially greater depth of cooling (30°C), which may confer some protection, but this depth is not likely to be used in the clinic as most clinical protocols and studies use a much milder depth (32-35°C).[15] Gao and others[13] used a depth similar to ours and found a benefit days after rewarming. This is contrary to what we found despite our use of a much longer duration of cooling and a slower rewarming, which are both typically considered to be more beneficial. This discrepancy is difficult to explain although there are a couple key differences. Gao and others[13] induced TH immediately after insult while we delayed our treatment by an hour. This delay is more appropriate as there would likely be a lag in time before TH is initiated in the clinic. Also, the beneficial effects found by Gao and others[13] may have been transient and not lasted past their last

time point. While they found benefit up to 7 days after stroke, our results indicate that TH may have had a transient effect that is gone by 14 days. It should be noted, there is preliminary evidence that shorter durations of cooling, when matched for timing to initiate, can be more beneficial than longer periods;[16] therefore, it is possible that our longer duration of cooling counteracted any early benefits. This may help explain why Goa and others[13] found benefit while we did not. However, more investigation is needed to better understand optimal duration of cooling, especially in relation to when TH is initiated. The need for more research for determining best practices for using TH will be discussed more thoroughly later in this chapter. As previously mentioned, both groups did not evaluate the effect of their TH protocol on functional impairments or neuroprotection but instead focused on edema as a marker for improvement. Both Kawai and others[12] and Gao and others[13] found a reduction in edema when we could not, but differences in depth, duration and delay in initiating cooling along with different doses and types of thrombin make comparisons difficult.

The reductionist model used in this thesis has been used by others to isolate and better understand the toxic and degenerative effects thrombin has on brain tissue.[5-8] However, there are a couple of limitations with this model. First, there are potential interactions between thrombin and other mechanisms of injury following an ICH that are not accounted for which can either further enhance or mitigate the toxicity of thrombin. Regardless of whether TH would have an effect on these interactions, these results show that it does not influence the direct neurotoxic effects of thrombin. As well, our bolus infusion of thrombin would cause tissue to come in contact with thrombin at a faster pace than during a typical hemorrhage. However, as the data from Gao and others[13] show, there is an increase in edema from 6 to 24 hours following insult

and our protocol of initiating cooling at 1 hour post-infusion should have been able to mitigate that increase in edema.

6.2.2 Iron

Similar to our results in Chapter 2, experiments in Chapter 3 indicate that TH does not effectively target another key mechanism of injury – iron-induced neurotoxicity.[4] Iron infusion caused a small but significant amount of bleeding that cooling was able to mitigate; however, this did not translate to any other positive effects. There was no effect of TH on edema either early on (i.e., 24 hours post infusion) or later (i.e., 72 hours). As well, TH failed to provide neuroprotection or functional benefit at either 7 or 28 days following infusion. Several endpoints and time points were used to thoroughly evaluate whether TH may confer some protection against iron-induced injury but our results overwhelmingly suggest that iron-induced damage was not influenced by cooling.

Like the thrombin model, infusion of $FeCl_2$ to isolate the effects on iron-induced injury on the brain has limitations. In this model we used a bolus infusion of $FeCl_2$ into the striatum which does not model the degradation of erythrocytes and release of iron from hemoglobin that occurs over the days following a bleed.[9,17] But the model does allow us to better understand how cooling can influence iron-induced damage and others have used this reductionist approach to both understand iron-induced neurotoxicity and evaluate the influence of treatments on this damage.[11,18] Also, Caliaperumal and others[11] have shown that unlike the thrombin reductionist model, infusion of $FeCl_2$ causes chronic cell death with greater tissue loss at 60 compared to 7 days post-infusion. This suggests that there is on-going damage that our treatment protocol would be able to target making this a relevant model to study on-going damage caused by iron.

While we did not evaluate the mechanisms of iron-induced injury such as production of free radicals and oxidative injury, we did focus on functional outcomes and neuroprotection as they are the endpoints with more importance clinically.

6.3 Bipyridine

Although the majority of this thesis focused on cooling as a therapy, we also investigated an iron chelator, bipyridine, as a therapeutic intervention for ICH with the hope of using it in combination with TH. Most of the research on iron-chelators focuses on deferoxamine; however, previous work from our lab extensively studied deferoxamine and failed to find a benefit for hemorrhagic stroke.[19,20] Thus, we chose to study bipyridine as it has better BBB permeability, chelates the more volatile ferrous form of iron, and can chelate iron intracellularly.[18,21]

In Chapter 5, we extensively investigated bipyridine as a potential therapy for treating ICH with the hopes of then studying it in combination with TH if we found bipyridine to have beneficial effects on its own. Unfortunately, despite using three different animal models and varying the dosing regimen, we were unable to find any beneficial effects of bipyridine for hemorrhagic stroke.[22] To ensure that our solution of bipyridine was active and chelating iron, we also conducted a spectrometry experiment. Our results confirmed that the bipyridine used in our experiments was binding iron at a 3:1 mole ratio as expected. We used the collagenase model of ICH in two separate studies assessing the effectiveness of bipyridine as an iron chelator. The first study followed a previously published protocol that included a higher dose of bipyridine given more frequently;[18] however, we found that this dose caused significant weight loss and feces discolouration, which is indicative of bipyridine toxicity.[23] The second study used a lower dose of bipyridine given less frequently but earlier after the stroke, as we followed the drug regimen

of a newer study that was published after our first experiment and did not state any side effects.[24] There was still significant weight loss caused by bipyridine with the lower dosage, but the loss was not as dramatic and there was no discolouration of the feces. We considered this dose regimen to be safer and used it in the subsequent study using the whole-blood model of ICH. Neither experiment that used the collagenase model found any effect on behavioural outcomes, non-heme iron levels, or edema.

Our experiment using the whole-blood model also did not detect any functional or neuroprotective effects of bipyridine. The two experiments using the smaller dose of bipyridine did find that there was a transient decrease in body temperature after each injection. Cooling is known to decrease edema following stroke[1,2] and if the animals were still cooled during euthanasia this could explain the reduction of edema found in the previous study.[24] In our experiment, animals were euthanized for edema measurement after their body temperature had returned to normothermia, which may explain why we did not find an effect of bipyridine on edema. Finally, we studied whether pre-treatment of bipyridine could mitigate the effects of iron-induced damage by using the same $FeCl_2$ model used in Chapter 3. We used the higher dose that was used in our first collagenase experiment but began treatment two hours prior to $FeCl_2$ to ensure there was adequate drug to chelate the bolus injection of iron. Unfortunately, even with a pre-treatment at a higher and more frequent dose post-$FeCl_2$ infusion, we did not find any protective effects of bipyridine. Additionally, as pre-treated animals exhibited inexplicable abnormal motor movements in the first hour following $FeCl_2$ infusion, all other experiments using $FeCl_2$ and bipyridine were cancelled.

Our study of bipyridine focused on histological and functional endpoints while others have also studied mechanistic effects of bipyridine. Nakamura and colleagues[18] found that

bipyridine was able to decrease markers of oxidative stress; however, they did not evaluate functional impairment and neuroprotection, which are more clinically relevant. Since we did not find any positive effect on any histological or functional endpoints, we did not see a need to carry out a mechanistic study.

Wu and others[24] did extensively study the effects of bipyridine on ICH by using both the collagenase and whole-blood model of ICH, using several endpoints and using both a pre-treatment and post-treatment approach. They found that pre-treatment significantly reduced iron levels, neuronal degeneration and oxidative stress following a collagenase-induced ICH. As well, in their study, bipyridine post-treatment reduced lesion volume, behavioural impairment, and edema at 3 days following a collagenase-induced hemorrhage. We were unable to replicate these results in our collagenase model experiments; however, there are a few differences between studies. Wu and others[24] evaluated lesion volume at 3 days post-ICH, which was soon after they finished giving their treatment. Conversely, we evaluated tissue loss and behavioural deficits several days after post-treatment. There is the possibility that the neuroprotective effects they found were transient and no longer present once treatment ended. As previously mentioned, bipyridine neuroprotection and effect on edema could be due to the transient cooling effect of bipyridine that we found and animals may have been euthanized while still cold in their study. Unfortunately, this does not explain why they did find functional benefit 4 weeks post-ICH and we could not at 1 week. We did do a more thorough evaluation with three behavioural tests (as opposed to their one test) and there was a difference in dosing and injection schedule between the two studies. The experiments involving the whole-blood model are better matched between Wu and others[24] and our experiment in terms of using the same dose and injection schedule. In the whole-blood model, Wu and others[24] found that there was an effect on edema as well as on

behavioural deficits at 3 days post-ICH. We evaluated the effect of bipyridine on behaviour and neuroprotection at 7 days-post ICH and failed to find an effect. Again, there is a difference in the time at which endpoints were conducted and it is possible that the behavioural protection they found was transient. As well there was a species difference between studies; we used rats in our experiments and Wu and others[24] used mice.

As with deferoxamine it is difficult to compare across studies due to differences in dosing, beginning of treatment and duration, and use of different species. However, despite using different dose regimens and three different models of injury, we were unable to find benefit on a single endpoint. We do know the bipyridine we used was having an effect as there was a significant drop in body temperature after each injection and side effects (e.g., weight loss, feces discolouration). In our lab we were also unable to find an effect of deferoxamine in preclinical ICH studies.[20] Recently, a phase II clinical trial where deferoxamine was tested as a treatment for ICH has been published, and despite a large amount of successful preclinical literature, deferoxamine did not pass this clinical trial. It is difficult to fund and publish negative studies, however, these types of preclinical studies are important to help provide accurate information when determining which drugs should be translated to the clinic. Our results help show the need for publishing negative studies.

We did not evaluate whether a combination of bipyridine and cooling would be beneficial for ICH. There is some evidence that TH can be neuroprotective against a hemorrhage and there is robust evidence that it reduces inflammation, edema, and BBB damage, which is also seen in preclinical ischemia literature.[1,2] Our results from Chapter 3 suggest that iron toxicity is a mechanism that TH does not mitigate that would benefit from a pharmacological therapy that does target iron. Yet in our opinion, there is insufficient evidence that bipyridine provides

enough, if any, protection on its own to warrant a combination therapy study. In a metal-analysis to determine a potential combination therapy to experimentally study, O'Collins and others[25] required that each therapy needed to demonstrate efficacy on their own. We were unable to find benefit on any endpoint despite using multiple measures and models for bipyridine including an iron reductionist model. Therefore, while our previous studies showed that TH does not target iron induced injury, we did not deem bipyridine as a suitable pharmacological agent for combination therapy. It is possible that deferoxamine would be a more suitable drug to use in a combination therapy; however, in our lab we did not find any benefit of deferoxamine despite using several endpoints. As well, I will review the results from our last study in the following section that suggests that there are other more significant complications of using TH to treat ICH beyond failure to protect against iron-induced damage.

6.4 Potential Side Effects of Therapeutic Hypothermia

As with any therapy there is potential for TH to have negative side effects. Well known side effects include pneumonia, as well as, rebound edema and ICP with rapid rates of rewarming. However, we hypothesized that TH had potential negative effects that were specific to ICH. In both ICH and ischemic stroke, there is neuronal and glial cell death either from primary damage of blood tearing through tissue or necrotic and apoptotic cell death induced by oxygen deprivation, respectively. However, in ICH inflammatory cells have the extra demand of containing and resolving blood components, which is why we hypothesized that the reduction in inflammation would hinder the endogenous mechanisms for hematoma containment and resolution. The inflammatory response following an ICH is important to the containment and resolution of the hematoma as its components contribute to ongoing cell death (e.g., release of

iron from erythrocytes propagates oxidative stress). Glial cells help contain the blood from further spreading to and affecting surrounding tissue. Inflammatory cells also aid in hematoma resolution by engulfing the constituents of the hematoma, preventing extracellular release of toxic factors (e.g., iron), and producing proteins that nullify potential toxins (e.g., production of ferritin and transferrin to bind to iron and prevent it from catalyzing oxidative stress).[9,17] While our results did not support our hypothesis, we did find rebleeding, in a subset of animals, which was an unexpected yet important side effect.

In Chapter 4, the first experiment used synchrotron imaging to spatially map iron levels in the brain of rats following a collagenase-induced hemorrhage. We did find a significant relationship between iron levels and the distance from the edge of the hematoma, with iron levels decreasing further away from the hematoma, but there was no effect of TH on the spread of iron from the hematoma. In this experiment we also confirmed that TH significantly reduces the number of inflammatory cells. In the second experiment, we assessed non-heme iron levels at 3 and 7 days following a collagenase-induced ICH to indirectly evaluate the effect of TH on hematoma resolution. There was an increase in non-heme iron levels from 3 to 7 days post-stroke in the injured forebrain. This increase is expected as erythrocytes and hemoglobin break down over the course of days following the initial bleed, but we did not find differences between normothermic and hypothermic animals at either time. Thus, from this measure, it does not appear that TH influences hematoma resolution.

We also evaluated functional impairments at a week post-stroke but did not find an effect of TH. To further investigate the impact of TH on hematoma resolution, we conducted a third experiment measuring blood volume at 1, 3 and 7 days following collagenase-induced ICH. We found that there was a significantly large increase in blood volume in TH treated animals at 7

days post-ICH, but no differences between treated and untreated groups at either day 1 or 3. As seen in Figure 7 of Chapter 4, there is a bimodal distribution in blood volume levels in the treated group, with treatment causing a substantial increase in blood volume in only a subset (i.e., 40%) of animals. We think this increase in blood volume is due to a rebleeding following treatment for the following reasons. First, there was no difference in blood volume between treated animals at 1 and 3 days, which is when animals were still under treatment and were euthanized cold. Moreover, experimenter observations at the time of euthanasia noted brighter red colouring of blood seen in the larger bleeds instead of the rust colouring typical of older bleeds. Since this effect is only seen in a subgroup of animals and has not been documented before, this rebleeding effect needs to be replicated.

The rebleeding results could help explain why there is inconsistent benefit with TH treatment. Since not all treated animals experienced this increase in blood volume, the proportion of animals that do will affect the ability to detect a statistically significant neuroprotective effect. In the third experiment, we did find that treated animals were slightly (1 point difference in median scores) but significantly more impaired than untreated animals at a week post-ICH, but we were unable to correlate behavioural scores with blood volume. Therefore, slight differences in behavioural impairments are not necessarily the best indicators of hemorrhage size and experiments that failed to find neuroprotective effects of TH for ICH may not necessarily also be able to also detect behavioural effects.

The cause of the rebleeding we found in our experiment is unclear. While it is known that treating ICH with TH too early can aggravate bleeding[26,27] we initiated cooling 12 hours post-collagenase infusion, which is known to be a safe delay,[26] and animals euthanized at earlier timepoints did not experience an increase in blood volume. It should be noted that it is unclear

whether TH caused rebleeding during rewarming or during the days following rewarming when animals returned to normothermia. The rate of rewarming is an important component when determining treatment parameters for TH. Rewarming too quickly can negate the protective effects provided by TH and exacerbate injury in both ischemic[28,29] and hemorrhagic stroke.[30] The rewarming rate of 0.5°C/hour, which is slower than the rate used in previous studies we based our TH protocol on,[26,31] was used in our experiments as we opted for a more conservative approach to rewarming. As previously mentioned, passive or fast rewarming can have negative effects (e.g., rebound edema, impaired cerebrovascular responsiveness) compared to slow rewarming.[30,32,33] As well, since rebleeding was seen in only a subset of animals, it is unlikely that rewarming alone would have been the cause, although this is not clear from our results.

It is possible that the rebleeding was caused by either changes in BP, cerebral blood flow at the site of injury, a stress response, or a combination of different factors. More recent work from our lab (Dietrich and Colbourne unpublished) sought to replicate this rebleeding post-treatment and assess the impact of TH on BP and hematoma size. None of their animals experienced a significant rebleeding at a week post-stroke despite using the same treatment protocol, suggesting that the occurrence is less frequent than the 40% proportion of animals found in our study. The inconsistency of this rebleeding suggests large sample sizes are needed to appropriately assess this event. Interestingly, they did find a statistically significant and close relationship between BP and hematoma size. During the rewarming and post-treatment phases, the more time that BP was higher than baseline, BP was related to a larger hematoma blood volume. This association during the rewarming and post-treatment phases aligns with when the rebleeding would have occurred in our study. They also found that this effect was only significant when comparing to the individual rat's baseline BP, and the effect was lost when a

group baseline average was used, demonstrating that there can be substantial variability in BP and vasculature in normal rats. This natural variability could explain the variability in hematoma size and the inconsistency of rebleeding. While they did not have any rats with significant rebleeding, such as the quadruple increase in blood volume that we observed, their results showed the importance of BP post-stroke and hematoma size, particularly during the rewarming and post-rewarming phases, as well as the influence of an individual's BP and vasculature on hematoma size. Other studies have shown that in animal models there is ongoing BBB disruption in the days following ICH with a peak in leakage at 3 days that subsides by day 7[34,35] and in a subset of rats there is ongoing dysfunction up to 14 days.[35] This on-going BBB leakage and in particular the long-term disruption may make certain animals more susceptible to the effects of rewarming.

While the research mentioned above suggests that BP and BBB dysfunction are possible explanations on why rebleeding occurs in a subset of animals, it does not definitively prove so. There may be other factors, such as changes in blood flow at the site of injury and/or regional perfusion pressure, that have not been evaluated and more research is needed. One small clinical trial for ICH found that TH caused increases in regional cerebral blood flow,[36] and while they found TH to also be beneficial, it is possible that in those with greater BBB dysfunction this increase in regional blood flow could be harmful. It is also possible that there are other side effects of using TH to treat ICH that were not detected in these experiments and, as our results were unexpected, they highlight the need to better understand how TH affects ICH. There may also be negative effects on hematoma containment and resolution that were not detected by the methods we used and more research (e.g., use of Raman spectroscopy to more accurately

measure hemoglobin levels) would be needed to elucidate the anti-inflammatory effect of TH, and how this influences the hematoma.

6.5 Translational Issues

Despite decades of research on the mechanisms of injury after ICH and potential therapies, no neuroprotective therapy has translated to the clinic. The issue of translating preclinical research to clinical research and application is complex and extends beyond stroke research. For instance, scientific integrity, experimental design and replication are essential to all scientific progress.[37,38] Within preclinical stroke research, there are also issues around animal models, inclusion of co-morbidities, etc.[39,40]

There is no one culprit behind why preclinical research is not meeting the success we want in translation. This issue involves funders, publishers, reviewers, the public and researchers who all participate in this issue more often than not with the good intentions of meeting the health needs of humans. After all, this is the main goal of preclinical and clinical researchers. Pressures around funding and publishing, the publish or perish dilemma, and valuing quantity over quality of research has made it difficult to conduct and publish high quality research, negative research results and replication studies.[37] There is literature available that reporting on, or lack thereof, blinding influences the efficacy and reproducibility of preclinical research.[37,39] Efforts to replicate the results of published research, including studies in high impact journals, have failed.[37] If results are not reproducible in preclinical research it seems unlikely they will be reproduced in clinical trials. As well, the lack of publishing negative results (i.e., that a treatment did not work) has also been highlighted as a major issue[37] and can create an incomplete and

biased picture when determining which treatments to fund in clinical trials. For a more detailed review on challenges in preclinical research please refer to Begley and Ioannidis[37] and MacLeod and others.[38]

Over the past two decades, measures have been developed to improve the quality of stroke research including the ARRIVE guidelines for animal research[40] and the Stroke Therapy Academic Industry Roundtable (STAIR) recommendations.[41] As highlighted in these reports, reporting on blinding, group allocation concealment, sample size calculations and power, among other details can improve the quality of stroke research. In fact, as Schlattmann and Dirnagl[42] point out, through improper statistical analysis alone, the positive effects of a treatment can be inflated by the number of tests conducted. Without proper adjustments for multiple comparisons, inclusion of power analysis and proper sample sizes this may impact preclinical experiments. As well, in stroke preclinical research most studies are carried out on young, healthy and male rodents. These reports also highlight the need for experiments on older animals and/or those with comorbidities commonly seen in the human stroke population (e.g., hypertension, diabetes, etc.) as well as the need to study treatment in both male and female animals.[39] It is important to thoroughly evaluate treatments preclinically in models that more accurately reflect the typical stroke population. For instance, as the stroke typically occurs in older adults, treatments should be evaluated in older animals and/or in animal with comorbidities, such as hypertension, as these factors can impact recovery and mitigate positive effects of a treatment.[39] As well, most preclinical literature used male rodents; yet, there are differences in incidence and outcome between males and females.[43] While males are more likely to have an ICH, women tend to have worse functional impairments despite no differences in hematoma volume; thus highlighting the need to do preclinical studies in both male and female animals.[43] Experiments with rodent

models of injury are by far more common than other animal models; however, there are anatomical differences between rodents and humans. For example, rodents have far less white matter than humans making rodents less suitable for testing effects on white matter damage.[39] Porcine or non-human primate models of injury would more accurately reflect the damage of brain injury on white matter and the effects of a therapeutic on this damage.[39] Multiple endpoints on both histological and functional measures as well as testing in multiple models of stroke are also needed.[39] For a more in-depth look into the recommendations and guidelines for preclinical research please see Fisher and others' updated STAIR report[39] and the ARRIVE guidelines developed by Kilkenny and others.[40]

With the help of tools such as the ARRIVE guidelines and STAIR recommendations as well as programs like the Neuroscience Information Network and Canadian Equator Centre that educate on and support improved standards for preclinical research, preclinical research will improve and hopefully lead to better translation to clinical trials. Even within this thesis we did not include power analysis and sample size calculations until our last publication (Chapter 4). While we did base our group sizes on previous work which often included n's larger than the studies we attempted to replicate, this is still a limitation on our part, and we recommend sharing sample size calculations and conducting power analyses in future studies. Also, while we did attempted to conduct behavioural testing during the same time each day, we did not standardize this, nor did we standardize the order of testing, both of which can contribute to variability in behavioural data making it more difficult to detect potential effects. Other improvements in behavioural testing such as including timing of crossing on the horizontal ladder tests could have improved our testing procedures; however, as there were no histological effects in most of our experiments we do not think there were missed behavioural effects. Issues around research

translation are difficult to resolve but necessary to address and will involve participation from all parties including researchers, editors, reviewers and funders.

6.6 Future Directions

Our results demonstrating that TH does not mitigate iron-induced damage suggest that this would be a good target for a pharmacological combination therapy with TH. However, as previously mentioned, as we did not find any benefit of bipyridine on any endpoint we did not deem bipyridine to be a suitable drug for combination therapy. This is unfortunate as there has been considerable attention on iron-chelation as a potential therapy for ICH. Nevertheless, despite the mostly positive animal research on deferoxamine, a phase II clinical trail failed to find benefit.[44] Preclinical studies that fail to find neuroprotection and behavioural improvement, such as our experiments on bipyridine[22] and deferoxamine,[20] are important in the translation of therapies from the lab to the clinic. Moreover, the failure of translation of iron-chelators for ICH demonstrate the need for improvement in future studies. This improvement may include using more clinically relevant endpoints and time windows, having greater statistical power, experimenting in older animals and/or animals with comorbidities, and publication of negative results in preclinical studies.[45]

Our failure to find benefit with iron chelation therapy does not discount other potential drugs that target oxidative stress or clotting, such as tranexamic acid which is currently under clinical trial,[46] but further research would be needed to evaluate combination therapies. In other areas of brain injury, the combination of xenon and TH is being investigated for treating neonatal hypoxia with the hope of having enhanced neuroprotection;[47] however, a recent clinical trial

found this combination to be safe but did not provide additional benefit.[48] As there is lack of preclinical literature on using xenon as a treatment for ICH we did not focus on this treatment as a combination therapy in this thesis. Based on the results from this thesis, thrombin is another potential target, but it is not easy to find a suitable drug to mitigate thrombin as thrombin is important for clot formation and angiogenesis. Most studies have looked at enhancing clot formation through thrombin generation. For instance, administration of recombinant factor VIIa (rFVIIa) to promote clot formation was found to significantly reduce hematoma growth in patients but this unfortunately did not correspond to functional improvement.[49] This lack of functional benefit may be due to the neurotoxicity from over generation of thrombin, or that the reduction in hematoma volume was not large enough to lead to functional improvement. Even when rFVIIa was used on those who were more likely to have further hematoma expansion (i.e., a positive spot sign on computed tomography angiography), there was no benefit provided at 3 months post-stroke.[50] Others in our lab have studied novel rFVIIa analogs (Williamson and Colbourne unpublished) but were unable to find a suitable alternative. Thus, the complexity of thrombin's role in preventing hematoma expansion but also contributing to damage make it a difficult target.

The most surprising result from this thesis is the rebleeding that occurs in a subset of rats following TH. The variability of this rebleeding event, and that this is the first published literature demonstrating this complication, means that other studies are needed to better understand the proportion of animals affected, the cause, and potential ways to prevent rebleeding. Replication is an essential part of research that should be done for all studies and, unfortunately, the pressure to publish high impact research in high impact journals does not encourage the funding, completion, and publishing of replication studies.[37] Still, if further

research into using TH for treating ICH is to be conducted, complications such as rebleeding need to be better evaluated.

A combination therapy of TH and a pharmaceutical may not be necessary to avoid the rebleeding complication. As previously mentioned, results from Nadeau and others[35] suggest that some animals have on-going BBB dysfunction. As well, other recent work (Dietrich and Colbourne unpublished) shows that there is an association between the duration of BP elevated above baseline during the rewarming and post-rewarming phase and hematoma size. It may be that certain animals with greater BBB dysfunction and prolonged increases in BP are at a greater risk of rebleeding. It is unlikely that BBB dysfunction alone causes the rebleeding, as Nadeau and others[35] found that even when warfarin was given during peak BBB dysfunction, the barrier was intact enough to prevent further bleeding in the brain. It may be that multiple factors (e.g., BP changes, BBB disruption) are involved and when acting together create the capacity for rebleeding following TH. Further research would be needed to confirm this and identify all factors, but simply varying the treatment protocol for TH may avoid the rebleeding complication. It is possible that increasing the duration of TH to match the length of BBB dysfunction and prolonging the rate of rewarming to mitigate increases in BP may avoid the occurrence of rebleeding. However, a recent preclinical study by Lyden and others[16] found that, when matched for time to initiating treatment, shorter durations of TH were more neuroprotective following ischemic stroke in rats than longer durations. Their study found that longer durations of TH could negatively impact astrocyte support for the BBB. It should be noted, their method of cooling involved anesthesia throughout the cooling treatment and differing durations of anesthesia may confound their results. As well, their investigation into potential mechanism was done *in vitro* and more studies using animal models are needed to both confirm and examine this

effect. Thus, a well-designed study varying treatment parameters should be conducted to identify a better treatment protocol specifically for ICH to prevent rebleeding complications.

6.7 Summary

Ultimately, our goal was to better understand the effects of TH on mechanisms of secondary damage after ICH. Recognizing the weaknesses of TH allows for identifying potential targets for a pharmacological therapy which can be used in combination with TH for enhanced protection after ICH. While we did gain a better understanding of how TH affects secondary mechanisms of injury after ICH, we did not find a suitable candidate for a combination therapy. Most importantly though, we did find a surprising side effect of using TH to treat ICH – a rebleeding in a subset of animals that occurs either during rewarming or post-treatment. Although this rebleeding does not occur in all subjects, the substantial increase in hematoma volume is important as hematoma size is a predictor of outcome following ICH.[51]

Frustratingly, there have been no neuroprotective treatments approved for ICH patients despite decades of research. The success of using TH to treat global ischemia and infant HIE brought hope for protection in stroke. Even for ischemic stroke, where TH has been more extensively studied, clinical trials are not demonstrating the same level efficacy found in the preclinical literature. These recent clinical trials have failed to gain the necessary statistical power to determine efficacy.[52,53] Multisite clinical trials for hemorrhagic stroke are currently underway but have not been completed or updated in recent years.[54,55] Unfortunately, the studies from this thesis suggest that unless the rebleeding complication can be mitigated, TH may not be a suitable treatment for ICH patients. Although a subset of patients with high levels of ICP may

benefit from a reduction in pressure, this effect is minor and there is no strong evidence that TH reduces mortality or improves outcome following ICH.[15] Thus, from the results of this thesis and the lack of a more consistent neuroprotective and/or functional beneficial effect in other preclinical ICH studies, we do not recommend using TH to treat ICH without further preclinical research on potential complications. As discouraging as these results are, there is continued research being done on best management practices to treat ICH patients, such as managing BP.[56] As well, the effects of rehabilitation, which appear to be especially effective for hemorrhagic stroke,[57,58] have robust evidence that stems from animal studies.[59-61] While there are difficulties in translating preclinical stroke research, animal studies can still add valuable insights through thorough investigation of the efficacy, mechanisms of action, and complications of potential treatments.

6.8 References

1. MacLellan CL, Clark DL, Silasi G, Colbourne F. Use of prolonged hypothermia to treat ischemic and hemorrhagic stroke. J Neurotrauma 2009 Mar;26(3):313-323.

2. Melmed KR, Lyden PD. Meta-analysis of pre-clinical trials of therapeutic hypothermia for intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2017 Sep;7(3):141-146.

3. Wowk S, Ma Y, Colbourne F. Mild therapeutic hypothermia does not reduce thrombin-induced brain injury. Ther Hypothermia Temp Manag 2014 Dec;4(4):180-187.

4. Wowk S, Ma Y, Colbourne F. Therapeutic hypothermia does not mitigate iron-induced injury in rat. Ther Hypothermia Temp Manag 2016 Mar;6(1):23-29.

5. Caliaperumal J, Brodie S, Ma Y, Colbourne F. Thrombin causes neuronal atrophy and acute but not chronic cell death. Can J Neurol Sci 2014 Nov;41(6):714-720.

6. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

7. Lee KR, Kawai N, Kim S, Sagher O, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: Effects of thrombin on cerebral blood flow, blood-brain barrier permeability, and cell survival in a rat model. Journal of neurosurgery 1997 Feb;86(2):272.

8. Lee KR, Drury I, Vitarbo E, Hoff JT. Seizures induced by intracerebral injection of thrombin: A model of intracerebral hemorrhage. Journal of neurosurgery 1997 Jul;87(1):73-78.

9. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke 2003 Dec 1,;34(12):2964-2969.

10. Wu G, Xi G, Hua Y, Sagher O. T2* magnetic resonance imaging sequences reflect brain tissue iron deposition following intracerebral hemorrhage. Transl Stroke Res 2010 Mar 01,;1(1):31-34.

11. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

12. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

13. GAO D, DING F, LEI G, LUAN G, ZHANG S, LI K, et al. Effects of focal mild hypothermia on thrombin-induced brain edema formation and the expression of protease activated receptor-1, matrix metalloproteinase-9 and aquaporin 4 in rats. Molecular Medicine Reports 2015 Apr;11(4):3009-3014.

14. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

15. Yao Z, You C, He M. Effect and feasibility of therapeutic hypothermia in patients with hemorrhagic stroke: A Systematic review and meta-analysis. World Neurosurgery 2018 Mar;111:404-412.e2.

16. Lyden PD, Lamb J, Kothari S, Toossi S, Boitano P, Rajput PS. Differential effects of hypothermia on neurovascular unit determine protective or toxic results: Toward optimized therapeutic hypothermia. Journal of Cerebral Blood Flow & Metabolism 2019 Sep;39(9):1693-1709.

17. Wan J, Ren H, Wang J. Iron toxicity, lipid peroxidation and ferroptosis after intracerebral haemorrhage. Stroke and vascular neurology 2019 Jul;4(2):93-95.

18. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

19. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

20. Warkentin LM, Auriat AM, Wowk S, Colbourne F. Failure of deferoxamine, an iron chelator, to improve outcome after collagenase-induced intracerebral hemorrhage in rats. Brain Research 2009;1309:95-103.

21. Bridges KR, Cudkowicz A. Effect of iron chelators on the transferrin receptor in K562 cells. The Journal of Biological Chemistry 1984 November;259(21):12970-12977.

22. Caliaperumal J, Wowk S, Jones S, Ma Y, Colbourne F. Bipyridine, an iron chelator, does not lessen intracerebral iron-induced damage or improve outcome after intracerebral hemorrhagic stroke in rats. Transl Stroke Res 2013 Dec;4(6):719-728.

23. Groce DF, Kimbrough RD. Acute and subacute toxicity in sherman strain rats exposed to 4,4'- and 2,2'-dipyridyl. Journal of Toxicology and Environmental Health 1982 Sep 1,;10(3):363-372.

24. Wu H, Wu T, Li M, Wang J. Efficacy of the lipid-soluble iron chelator 2,2'-dipyridyl against hemorrhagic brain injury. Neurobiol Dis 2012 Jan;45(1):388-394.

25. O'Collins VE, Macleod MR, Cox SF, Van Raay L, Aleksoska E, Donnan GA, et al. Preclinical drug evaluation for combination therapy in acute stroke using systematic review, meta-analysis, and subsequent experimental testing. J Cereb Blood Flow Metab 2011 Mar;31(3):962-975.

26. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

27. John RF, Williamson MR, Dietrich K, Colbourne F. Localized hypothermia aggravates bleeding in the collagenase model of intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2015 Mar 1,;5(1):19-25.

28. Berger C, Xia F, Köhrmann M, Schwab S. Hypothermia in acute stroke--slow versus fast rewarming an experimental study in rats. Exp Neurol 2007 Mar;204(1):131-137.

29. Steiner T, Friede T, Aschoff A, Schellinger PD, Schwab S, Hacke W. Effect and feasibility of controlled rewarming after moderate hypothermia in stroke patients with malignant infarction of the middle cerebral artery. Stroke 2001 Dec 1,;32(12):2833-2835.

30. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

31. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

32. Nakamura T, Miyamoto O, Yamagami S, Hayashida Y, Itano T, Nagao S. Influence of rewarming conditions after hypothermia in gerbils with transient forebrain ischemia. J Neurosurg 1999 Jul;91(1):114-120.

33. Ueda Y, Suehiro E, Wei EP, Kontos HA, Povlishock JT. Uncomplicated rapid posthypothermic rewarming alters cerebrovascular responsiveness. Stroke 2004 Feb 1,;35(2):601-606.

34. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

35. Nadeau C, Nadeau C, Dietrich K, Dietrich K, Wilkinson C, Wilkinson C, et al. Prolonged blood-brain barrier injury occurs after experimental intracerebral hemorrhage and is not acutely associated with additional bleeding. Transl Stroke Res 2019 Jun 15,;10(3):287-297.

36. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

37. Begley C, Ioannidis J. Reproducibility in science: Improving the standard for basic and preclinical research. Circulation Research 2015 Jan 2,;116(1):116-126.

38. Macleod MR, Fisher M, O'Collins V, Sena ES, Dirnagl U, Bath PMW, et al. Good laboratory practice: Preventing introduction of bias at the bench. Stroke 2009 Mar;40(3):50.

39. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, et al. Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke 2009 Jun;40(6):2244-2250.

40. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: Reporting in vivo experiments: The ARRIVE guidelines. British Journal of Pharmacology 2010 Aug;160(7):1577-1579.

41. Stroke Therapy Academic Industry Roundtable. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 1999 Dec;30(12):2752-2758.

42. Schlattmann P, Dirnagl U. Statistics in experimental cerebrovascular research-comparison of two groups with a continuous outcome variable. Journal of Cerebral Blood Flow & Metabolism 2010 Mar;30(3):474-479.

43. Roquer J, Rodríguez-Campello A, Jiménez-Conde J, Cuadrado-Godia E, Giralt-Steinhauer E, Vivanco Hidalgo RM, et al. Sex-related differences in primary intracerebral hemorrhage. Neurology 2016 Jul 19,;87(3):257-262.

44. Selim M, Foster LD, Moy CS, Xi G, Hill MD, Morgenstern LB, et al. Deferoxamine mesylate in patients with intracerebral haemorrhage (i-DEF): A multicentre, randomised, placebo-controlled, double-blind phase 2 trial. The Lancet Neurology 2019 May;18(5):428-438.

45. Schmidt-Pogoda A, Bonberg N, Koecke MHM, Strecker J, Wellmann J, Bruckmann N, et al. Why most acute stroke studies are positive in animals but not in patients: A systematic comparison of preclinical, early phase, and phase 3 clinical trials of neuroprotective agents. Ann Neurol 2020 Jan;87(1):40-51.

46. Liu L, Wang Y, Meng X, Li N, Tan Y, Nie X, et al. Tranexamic acid for acute intracerebral hemorrhage growth predicted by spot sign trial: Rationale and design. International Journal of Stroke 2017 Apr;12(3):326-31.

47. Amer AR, Oorschot DE. Xenon combined with hypothermia in perinatal hypoxic-ischemic encephalopathy: A noble gas, a noble mission. Pediatr Neurol 2018;84:5-10.

48. Azzopardi D, Robertson NJ, Bainbridge A, Cady E, Charles-Edwards G, Deierl A, et al. Moderate hypothermia within 6 h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-xe): A proof-of-concept, open-label, randomised controlled trial. Lancet Neurol 2016 -2;15(2):145-153.

49. Mayer SA, Brun NC, Begtrup K, Broderick J, Davis S, Diringer MN, et al. Efficacy and safety of recombinant activated factor VII for acute intracerebral hemorrhage. N Engl J Med 2008 May 15,;358(20):2127-2137.

50. Gladstone DJ, Aviv RI, Demchuk AM, Hill MD, Thorpe KE, Khoury JC, et al. Effect of recombinant activated coagulation factor VII on hemorrhage expansion among patients with spot sign-positive acute intracerebral hemorrhage: The SPOTLIGHT and STOP-IT randomized clinical trials. JAMA neurology 2019 Aug 19,;76(12):1493.

51. Davis SM, Broderick J, Hennerici M, Brun NC, Diringer MN, Mayer SA, et al. Hematoma growth is a determinant of mortality and poor outcome after intracerebral hemorrhage. Neurology 2006 Apr 25,;66(8):1175-1181.

52. Lyden P, Hemmen T, Grotta J, Rapp K, Ernstrom K, Rzesiewicz T, et al. Results of the ICTuS 2 trial (intravascular cooling in the treatment of stroke 2). Stroke 2016;47(12):2888-2895.

53. van der Worp, H Bart, Macleod MR, Bath PM, Bathula R, Christensen H, Colam B, et al. Therapeutic hypothermia for acute ischaemic stroke. results of a european multicentre, randomised, phase III clinical trial. European Stroke Journal 2019 Sep;4(3):254-262.

54. Kollmar R, Juettler E, Huttner HB, Dörfler A, Staykov D, Kallmuenzer B, et al. Cooling in intracerebral hemorrhage (CINCH) trial: Protocol of a randomized german-austrian clinical trial. Int J Stroke 2012 Feb;7(2):168-172.

55. Rincon F, Friedman DP, Bell R, Mayer SA, Bray PF. Targeted temperature management after intracerebral hemorrhage (TTM-ICH): Methodology of a prospective randomized clinical trial. Int J Stroke 2014 Jul;9(5):646-651.

56. Gioia L, Klahr A, Kate M, Buck B, Dowlatshahi D, Jeerakathil T, et al. The intracerebral hemorrhage acutely decreasing arterial pressure trial II (ICH ADAPT II) protocol. BMC neurology 2017 May 19,;17(1):100.

57. Okuda Y, Nakata T. Effect of intensive rehabilitation on improvement of activity of daily living after intracerebral hemorrhage: A retrospective observational study. International Journal of Rehabilitation Research 2019 Oct 29,:1.

58. Paolucci S, Antonucci G, Grasso MG, Bragoni M, Coiro P, De Angelis D, et al. Functional outcome of ischemic and hemorrhagic stroke patients after inpatient rehabilitation: A matched comparison. Stroke 2003 Dec;34(12):2861-2865.

59. Auriat AM, Wowk S, Colbourne F. Rehabilitation after intracerebral hemorrhage in rats improves recovery with enhanced dendritic complexity but no effect on cell proliferation. Behav Brain Res 2010 Dec 06,;214(1):42-47.

60. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

61. Williamson MR, Dietrich K, Hackett MJ, Caine S, Nadeau CA, Aziz JR, et al. Rehabilitation augments hematoma clearance and attenuates oxidative injury and ion dyshomeostasis after brain hemorrhage. Stroke 2017;48(1):195-203.

Bibliography

Chapter One

1. Heart and Stroke Foundation. Stroke in Canada[Internet]. Ottawa: Heart and Stroke Foundation, (2018, cited 2020 April 27); Available from: https://www.heartandstroke.ca/what-we-do/media-centre/news-releases/canadian-led-international-study-shows-huge-costs-of-delayed-access-to-stroke-care.

2. Bhalla A, Wang Y, Rudd A, Wolfe C. Differences in outcome and predictors between ischemic and intracerebral hemorrhage: The south london stroke register. Stroke 2013 Aug;44(8):2174-2181.

3. Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB. The stroke data bank: Design, methods, and baseline characteristics. Stroke 1988 May;19(5):547-554.

4. Broderick JP, Brott TG, Duldner JE, Tomsick T, Huster G. Volume of intracerebral hemorrhage. A powerful and easy-to-use predictor of 30-day mortality. Stroke 1993 Jul;24(7):987-993.

5. Navarrete-Navarro P, Hart WM, Lopez-Bastida J, Christensen MC. The societal costs of intracerebral hemorrhage in spain. European Journal of Neurology 2007 May;14(5):556-562.

6. Hemphill J, Greenberg S, Anderson C, Becker K, Bendok B, Cushman M, et al. Guidelines for the management of spontaneous intracerebral hemorrhage: A guideline for healthcare professionals from the american heart association/american stroke association. Stroke 2015 Jul;46(7):2032-2060.

7. Howard G, Cushman M, Howard VJ, Kissela BM, Kleindorfer DO, Moy CS, et al. Risk factors for intracerebral hemorrhage: The REasons for geographic and racial differences in stroke (REGARDS) study. Stroke 2013 May;44(5):1282-1287.

8. Martini SR, Flaherty ML, Brown WM, Haverbusch M, Comeau ME, Sauerbeck LR, et al. Risk factors for intracerebral hemorrhage differ according to hemorrhage location. Neurology 2012 Dec 4,;79(23):2275-2282.

9. Vonsattel JPG, Myers RH, Tessa Hedley-Whyte E, Ropper AH, Bird ED, Richardson EP. Cerebral amyloid angiopathy without and with cerebral hemorrhages: A comparative histological study. Annals of Neurology 1991 Nov;30(5):637-649.

10. Cavallini A, Fanucchi S, Persico A. Warfarin-associated intracerebral hemorrhage. Neurol Sci 2008 Sep;29(S2):266-268.

11. Dowlatshahi D, Butcher KS, Asdaghi N, Nahirniak S, Bernbaum ML, Giulivi A, et al. Poor prognosis in warfarin-associated intracranial hemorrhage despite anticoagulation reversal. Stroke 2012 Jul;43(7):1812-1817.

12. Flibotte JJ, Hagan N, O'Donnell J, Greenberg SM, Rosand J. Warfarin, hematoma expansion, and outcome of intracerebral hemorrhage. Neurology 2004 Sep 28,;63(6):1059-1064.

13. Brott T, Broderick J, Kothari R, Barsan W, Tomsick T, Sauerbeck L, et al. Early hemorrhage growth in patients with intracerebral hemorrhage. Stroke 1997 Jan;28(1):1-5.

14. Davis SM, Broderick J, Hennerici M, Brun NC, Diringer MN, Mayer SA, et al. Hematoma growth is a determinant of mortality and poor outcome after intracerebral hemorrhage. Neurology 2006 Apr 25,;66(8):1175-1181.

15. Wada R, Aviv RI, Fox AJ, Sahlas DJ, Gladstone DJ, Tomlinson G, et al. CT angiography "spot sign" predicts hematoma expansion in acute intracerebral hemorrhage. Stroke 2007 Apr;38(4):1257-1262.

16. Mayer SA, Brun NC, Begtrup K, Broderick J, Davis S, Diringer MN, et al. Efficacy and safety of recombinant activated factor VII for acute intracerebral hemorrhage. N Engl J Med 2008 May 15,;358(20):2127-2137.

17. Rincon F, Rincon F, Lyden P, Lyden P, Mayer S, Mayer S. Relationship between temperature, hematoma growth, and functional outcome after intracerebral hemorrhage. Neurocrit Care 2013 Feb;18(1):45-53.

18. MacLellan CL, Colbourne F. Mild to moderate hyperthermia does not worsen outcome after severe intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2005 August 1,;25(8):1020-1029.

19. Penner M, Silasi G, Wowk S, Warkentin L, Colbourne F. Brief hyperthermia does not worsen outcome after striatal hemorrhage in rats. Curr Neurovasc Res 2011 Feb;8(1):35-43.

20. Dandapani BK, Suzuki S, Kelley RE, Reyes-Iglesias Y, Duncan RC. Relation between blood pressure and outcome in intracerebral hemorrhage. Stroke 1995 Jan;26(1):21-24.

21. Anderson CS, Heeley E, Huang Y, Wang J, Wang J, Stapf C, et al. Rapid blood-pressure lowering in patients with acute intracerebral hemorrhage. The New England Journal of Medicine 2013 Jun 20,;368(25):2355-2365.

22. Qureshi AI, Palesch YY, Barsan WG, Hanley DF, Hsu CY, Martin RL, et al. Intensive blood-pressure lowering in patients with acute cerebral hemorrhage. The New England Journal of Medicine 2016 Sep 15,;375(11):1033-1043.

23. Leasure AC, Qureshi AI, Murthy SB, Kamel H, Goldstein JN, Woo D, et al. Association of intensive blood pressure reduction with risk of hematoma expansion in patients with deep intracerebral hemorrhage. JAMA neurology 2019 May 13,;76(8):949.

24. Menon RS, Burgess RE, Wing JJ, Gibbons MC, Shara NM, Fernandez S, et al. Predictors of highly prevalent brain ischemia in intracerebral hemorrhage: High prevalence of ischemic infarcts in ich. Annals of Neurology 2012 Feb 1,;71(2):199-205.

25. Gioia L, Kate M, Choi V, Sivakumar L, Jeerakathil T, Kosior J, et al. Ischemia in intracerebral hemorrhage is associated with leukoaraiosis and hematoma volume, not blood pressure reduction. Stroke 2015 Jun;46(6):1541-1547.

26. Prabhakaran S, Naidech AM. Ischemic brain injury after intracerebral hemorrhage: A critical review. Stroke 2012 Aug;43(8):2258-2263.

27. Butcher KS, Jeerakathil T, Hill M, Demchuk AM, Dowlatshahi D, Coutts SB, et al. The intracerebral hemorrhage acutely decreasing arterial pressure trial. Stroke 2013 Mar;44(3):620-626.

28. Gioia L, Klahr A, Kate M, Buck B, Dowlatshahi D, Jeerakathil T, et al. The intracerebral hemorrhage acutely decreasing arterial pressure trial II (ICH ADAPT II) protocol. BMC neurology 2017 May 19,;17(1):100.

29. Goldstein JN, Thomas SH, Frontiero V, Joseph A, Engel C, Snider R, et al. Timing of fresh frozen plasma administration and rapid correction of coagulopathy in warfarin-related intracerebral hemorrhage. Stroke 2006 Jan 1,;37(1):151.

30. Hinchey JA, Shephard T, Furie K, Smith D, Wang D, Tonn S, et al. Formal dysphagia screening protocols prevent pneumonia. Stroke 2005 Sep 1,;36(9):1972-1976.

31. Ko S, Choi HA, Parikh G, Helbok R, Schmidt JM, Lee K, et al. Multimodality monitoring for cerebral perfusion pressure optimization in comatose patients with intracerebral hemorrhage. Stroke 2011 Nov;42(11):3087-3092.

32. Keep RF, Xi G, Hua Y, Xiang J. Clot formation, vascular repair and hematoma resolution after ICH, a coordinating role for thrombin? Acta neurochirurgica. Supplement 2011;111:71-75.

33. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

34. Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. Prog Neurobiol 2010 Dec;92(4):463-477.

35. Caliaperumal J, Brodie S, Ma Y, Colbourne F. Thrombin causes neuronal atrophy and acute but not chronic cell death. Can J Neurol Sci 2014 Nov;41(6):714-720.

36. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

37. Power C, Henry S, Del Bigio MR, Larsen PH, Corbett D, Imai Y, et al. Intracerebral hemorrhage induces macrophage activation and matrix metalloproteinases. Annals of Neurology 2003 Jun;53(6):731-742.

38. Lee KR, Kawai N, Kim S, Sagher O, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: Effects of thrombin on cerebral blood flow, blood-brain barrier permeability, and cell survival in a rat model. Journal of neurosurgery 1997 Feb;86(2):272.

39. Yang S, Song S, Hua Y, Nakamura T, Keep RF, Xi G. Effects of thrombin on neurogenesis after intracerebral hemorrhage. Stroke 2008 Jul;39(7):2079-2084.

40. Yang A-, Zhou H, Lin Y, Luo J, Cui H, Tang T, et al. Thrombin promotes the expression of thrombospondin-1 and -2 in a rat model of intracerebral hemorrhage. J Neurol Sci 2012 Dec 15,;323(1-2):141-146.

41. Auriat AM, Wowk S, Colbourne F. Rehabilitation after intracerebral hemorrhage in rats improves recovery with enhanced dendritic complexity but no effect on cell proliferation. Behav Brain Res 2010 Dec 06,;214(1):42-47.

42. Hua Y, Keep RF, Gu Y, Xi G. Thrombin and brain recovery after intracerebral hemorrhage. Stroke 2009 Mar;40(3 Suppl):88.

43. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

44. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

45. Del Bigio MR, Yan HJ, Buist R, Peeling J. Experimental intracerebral hemorrhage in rats. magnetic resonance imaging and histopathological correlates. Stroke 1996 Dec;27(12):2312-2320.

46. Wasserman JK, Schlichter LC. Neuron death and inflammation in a rat model of intracerebral hemorrhage: Effects of delayed minocycline treatment. Brain Research 2007;1136(1):208-218.

47. Wasserman JK, Zhu X, Schlichter LC. Evolution of the inflammatory response in the brain following intracerebral hemorrhage and effects of delayed minocycline treatment. Brain Res 2007 Nov 14,;1180:140-154.

48. Xue M, Balasubramaniam J, Buist RJ, Peeling J, Del Bigio MR. Periventricular/intraventricular hemorrhage in neonatal mouse cerebrum. J Neuropathol Exp Neurol 2003 Nov;62(11):1154-1165.

49. Wu J, Yang S, Hua Y, Liu W, Keep RF, Xi G. Minocycline attenuates brain edema, brain atrophy and neurological deficits after intracerebral hemorrhage. Acta Neurochir Suppl 2010;106:147-150.

50. Zhao X, Sun G, Zhang J, Strong R, Song W, Gonzales N, et al. Hematoma resolution as a target for intracerebral hemorrhage treatment: Role for peroxisome proliferator-activated receptor gamma in microglia/macrophages. Ann Neurol 2007 Apr;61(4):352-362.

51. Zhao X, Grotta J, Gonzales N, Aronowski J. Hematoma resolution as a therapeutic target: The role of microglia/macrophages. Stroke 2009 Mar 1,;40(3, Supplement 1):S92-S94.

52. Zhao X, Sun G, Ting S, Song S, Zhang J, Edwards NJ, et al. Cleaning up after ICH: The role of Nrf2 in modulating microglia function and hematoma clearance. J Neurochem 2015 Apr;133(1):144-152.

53. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: Secondary brain injury. Stroke 2011 Jun;42(6):1781-1786.

54. Wagner KR, Sharp FR, Ardizzone TD, Lu A, Clark JF. Heme and iron metabolism: Role in cerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2003 Jun;23(6):629-652.

55. Hahl P, Davis T, Washburn C, Rogers JT, Smith A. Mechanisms of neuroprotection by hemopexin: Modeling the control of heme and iron homeostasis in brain neurons in inflammatory states. J Neurochem 2013 Apr;125(1):89-101.

56. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke 2003 Dec 1,;34(12):2964-2969.

57. Willmore LJ, Rubin JJ. Formation of malonaldehyde and focal brain edema induced by subpial injection of FeCl2 into rat isocortex. Brain research 1982 Aug 19,;246(1):113-119.

58. Wu J, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. Brain Res 2002 Oct 25,;953(1-2):45-52.

59. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

60. Katsu M, Niizuma K, Yoshioka H, Okami N, Sakata H, Chan PH. Hemoglobin-induced oxidative stress contributes to matrix metalloproteinase activation and blood-brain barrier dysfunction in vivo. J Cereb Blood Flow Metab 2010 Dec;30(12):1939-1950.

61. Nakamura T, Keep RF, Hua Y, Hoff JT, Xi G. Oxidative DNA injury after experimental intracerebral hemorrhage. Brain Res 2005 Mar 28,;1039(1-2):30-36.

62. Willmore LJ, Ballinger WE, Boggs W, Sypert GW, Rubin JJ. Dendritic alterations in rat isocortex within an iron-induced chronic epileptic focus. Neurosurgery 1980 Aug;7(2):142-146.

63. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

64. Hua Y, Nakamura T, Keep RF, Wu J, Schallert T, Hoff JT, et al. Long-term effects of experimental intracerebral hemorrhage: The role of iron. J Neurosurg 2006 Feb;104(2):305-312.

65. Kell DB. Towards a unifying, systems biology understanding of large-scale cellular death and destruction caused by poorly liganded iron: Parkinson's, huntington's, alzheimer's, prions, bactericides, chemical toxicology and others as examples. Arch Toxicol 2010 Nov;84(11):825-889.

66. Zhao X, Song S, Sun G, Strong R, Zhang J, Grotta JC, et al. Neuroprotective role of haptoglobin after intracerebral hemorrhage. J Neurosci 2009 Dec 16,;29(50):15819-15827.

67. Kell DB. Iron behaving badly: Inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC Med Genomics 2009 Jan 08,;2:2.

68. Wu G, Xi G, Hua Y, Sagher O. T2* magnetic resonance imaging sequences reflect brain tissue iron deposition following intracerebral hemorrhage. Transl Stroke Res 2010 Mar 01,;1(1):31-34.

69. Chen M, Awe OO, Chen-Roetling J, Regan RF. Iron regulatory protein-2 knockout increases perihematomal ferritin expression and cell viability after intracerebral hemorrhage. Brain Res 2010 -6-14;1337:95-103.

70. Grienberger HJ, Pillai DR, Schlachetzki F, Gruber M, Dittmar MS. Detection of free radicals by isolated perfusion of the rat brain following hemorrhagic stroke: A novel approach to cerebrovascular biomarker research. Exp Brain Res 2010 Oct;206(3):311-317.

71. 韩宁, Han N, Ding S, 丁素菊, Wu T, 吴涛, et al. Correlation of free radical level and apoptosis after intracerebral hemorrhage in rats. Neurosci Bull 2008 Dec;24(6):351-358.

72. Wang G, Yang Q, Li G, Wang L, Hu W, Tang Q, et al. Time course of heme oxygenase-1 and oxidative stress after experimental intracerebral hemorrhage. Acta Neurochir (Wien) 2011 Feb;153(2):319-325.

73. Papacocea T, Papacocea R, Badarau A, Ion AD, Burga I, Gaman L, et al. Oxidative stress and antioxidant therapy in intracerebral hemorrhage. Therapeutics, Pharmacology and Clinical Toxicology 2011 December;15(4):270-273.

74. Lyden PD, Shuaib A, Lees KR, Davalos A, Davis SM, Diener H, et al. Safety and tolerability of NXY-059 for acute intracerebral hemorrhage: The CHANT trial. Stroke 2007 Aug 1,;38(8):2262-2269.

75. Zafar A, Khan FS. Clinical and radiological features of intracerebral haemorrhage in hypertensive patients. J Pak Med Assoc 2008 Jul;58(7):356-358.

76. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

77. Bullock R, Mendelow AD, Teasdale GM, Graham DI. Intracranial haemorrhage induced at arterial pressure in the rat. part 1: Description of technique, ICP changes and neuropathological findings. Neurol Res 1984 Dec;6(4):184-188.

78. Rosenberg GA, Mun-Bryce S, Wesley M, Kornfeld M. Collagenase-induced intracerebral hemorrhage in rats. Stroke 1990 May;21(5):801-807.

79. Hiploylee C, Colbourne F. Intracranial pressure measured in freely moving rats for days after intracerebral hemorrhage. Exp Neurol 2014 May;255:49-55.

80. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

81. Klahr AC, Dickson CT, Colbourne F. Seizure activity occurs in the collagenase but not the blood infusion model of striatal hemorrhagic stroke in rats. Transl Stroke Res 2015 Feb;6(1):29-38.

82. Smeda JS. Hemorrhagic stroke development in spontaneously hypertensive rats fed a north american, japanese-style diet. Stroke 1989 Sep;20(9):1212-1218.

83. Lee J, Zhai G, Liu Q, Gonzales ER, Yin K, Yan P, et al. Vascular permeability precedes spontaneous intracerebral hemorrhage in stroke-prone spontaneously hypertensive rats. Stroke 2007 Dec;38(12):3289-3291.

84. Lee KR, Drury I, Vitarbo E, Hoff JT. Seizures induced by intracerebral injection of thrombin: A model of intracerebral hemorrhage. Journal of neurosurgery 1997 Jul;87(1):73-78.

85. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

86. Buchan A, Pulsinelli WA. Hypothermia but not the N-methyl-D-aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia. J Neurosci 1990 Jan;10(1):311-316.

87. Corbett D, Evans S, Thomas C, Wang D, Jonas RA. MK-801 reduced cerebral ischemic injury by inducing hypothermia. Brain Res 1990 Apr 30,;514(2):300-304.

88. Peliowski-Davidovich A. Hypothermia for newborns with hypoxic ischemic encephalopathy. Paediatrics & child health 2012 Jan;17(1):41-43.

89. Howes D, Gray SH, Brooks SC, Boyd JG, Djogovic D, Golan E, et al. Canadian guidelines for the use of targeted temperature management (therapeutic hypothermia) after cardiac arrest: A joint statement from the canadian critical care society (CCCS), canadian neurocritical care society (CNCCS), and the canadian critical care trials group (CCCTG). Resuscitation 2016 Jan;98:48-63.

90. González-Ibarra FP, Varon J, López-Meza EG. Therapeutic hypothermia: Critical review of the molecular mechanisms of action. Front Neurol 2011;2:4.

91. Karnatovskaia LV, Wartenberg KE, Freeman WD. Therapeutic hypothermia for neuroprotection: History, mechanisms, risks, and clinical applications. Neurohospitalist 2014 Jul;4(3):153-163.

92. Thomson J. Report of a case of sanguineous apoplexy successfully treated. The Lancet 1848 June /06;51(1293):634-635.

93. Howell DA, Posnikoff J, Stratford JG. Prolonged hypothermia in treatment of massive cerebral haemorrhage; a preliminary report. Canadian Medical Association journal 1956 Sep 1,;75(5):388-394.

94. The Hypothermia after Cardiac Arrest Study Group. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. The New England Journal of Medicine 2002 Feb 21,;346(8):549-556.

95. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med 2005 Oct 13,;353(15):1574-1584.

96. Schwab S, Schwarz S, Spranger M, Keller E, Bertram M, Hacke W. Moderate hypothermia in the treatment of patients with severe middle cerebral artery infarction. Stroke 1998 Dec;29(12):2461-2466.

97. The Brain Trauma Foundation. Guidelines for the management of severe traumatic brain injury. Journal of Neurotrauma 2007;24.

98. Colbourne F, Corbett D. Delayed postischemic hypothermia: A six month survival study using behavioral and histological assessments of neuroprotection. J Neurosci 1995 Nov;15(11):7250-7260.

99. Colbourne F, Li H, Buchan AM. Indefatigable CA1 sector neuroprotection with mild hypothermia induced 6 hours after severe forebrain ischemia in rats. J Cereb Blood Flow Metab 1999 Jul;19(7):742-749.

100. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

101. Colbourne F, Corbett D, Zhao Z, Yang J, Buchan AM. Prolonged but delayed postischemic hypothermia: A long-term outcome study in the rat middle cerebral artery occlusion model. J Cereb Blood Flow Metab 2000 Dec;20(12):1702-1708.

102. MacLellan CL, Clark DL, Silasi G, Colbourne F. Use of prolonged hypothermia to treat ischemic and hemorrhagic stroke. J Neurotrauma 2009 Mar;26(3):313-323.

103. Clark DL, Penner M, Wowk S, Orellana-Jordan I, Colbourne F. Treatments (12 and 48 h) with systemic and brain-selective hypothermia techniques after permanent focal cerebral ischemia in rat. Exp Neurol 2009 Dec;220(2):391-399.

104. Cooper DJ, Nichol AD, Bailey M, Bernard S, Cameron PA, Pili-Floury S, et al. Effect of early sustained prophylactic hypothermia on neurologic outcomes among patients with severe traumatic brain injury: The POLAR randomized clinical trial. JAMA 2018 12 04,;320(21):2211-2220.

105. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

106. Dididze M, Green BA, Dietrich WD, Vanni S, Wang MY, Levi AD. Systemic hypothermia in acute cervical spinal cord injury: A case-controlled study. Spinal cord 2013 May;51(5):395-400.

107. Abou-Chebl A, DeGeorgia MA, Andrefsky JC, Krieger DW. Technical refinements and drawbacks of a surface cooling technique for the treatment of severe acute ischemic stroke. Neurocrit Care 2004;1(2):131-143.

108. Georgiadis D, Schwarz S, Kollmar R, Schwab S. Endovascular cooling for moderate hypothermia in patients with acute stroke: First results of a novel approach. Stroke 2001 Nov;32(11):2550-2553.

109. Busch H-, Eichwede F, Födisch M, Taccone FS, Wöbker G, Schwab T, et al. Safety and feasibility of nasopharyngeal evaporative cooling in the emergency department setting in survivors of cardiac arrest. Resuscitation 2010 Aug;81(8):943-949.

110. Bernard SA, Smith K, Cameron P, Masci K, Taylor DM, Cooper DJ, et al. Induction of therapeutic hypothermia by paramedics after resuscitation from out-of-hospital ventricular fibrillation cardiac arrest: A randomized controlled trial. Circulation 2010 Aug 17,;122(7):737-742.

111. Cortez E, Panchal AR, Davis J, Zeeb P, Keseg DP. Clinical outcomes in cardiac arrest patients following prehospital treatment with therapeutic hypothermia. Prehosp Disaster Med 2015 Oct;30(5):452-456.

112. Hemmen TM, Raman R, Guluma KZ, Meyer BC, Gomes JA, Cruz-Flores S, et al. Intravenous thrombolysis plus hypothermia for acute treatment of ischemic stroke (ICTuS-L): Final results. Stroke 2010 Oct;41(10):2265-2270.

113. Lyden P, Hemmen T, Grotta J, Rapp K, Ernstrom K, Rzesiewicz T, et al. Results of the ICTuS 2 trial (intravascular cooling in the treatment of stroke 2). Stroke 2016;47(12):2888-2895.

114. NIELSEN N, HOVDENES J, NILSSON F, RUBERTSSON S, STAMMET P, SUNDE K, et al. Outcome, timing and adverse events in therapeutic hypothermia after out-of-hospital cardiac arrest. Acta Anaesthesiologica Scandinavica 2009 Aug;53(7):926-934.

115. Schwab S, Georgiadis D, Berrouschot J, Schellinger PD, Graffagnino C, Mayer SA. Feasibility and safety of moderate hypothermia after massive hemispheric infarction. Stroke 2001 Sep;32(9):2033-2035.

116. Wu T, Grotta JC. Hypothermia for acute ischaemic stroke. Lancet Neurol 2013 Mar;12(3):275-284.

117. Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, et al. Stroke-induced immunodepression: Experimental evidence and clinical relevance. Stroke 2007 Feb;38(2 Suppl):770-773.

118. Shi K, Wood K, Shi F, Wang X, Liu Q. Stroke-induced immunosuppression and poststroke infection. Stroke and Vascular Neurology 2018 Mar;3(1):34-41.

119. Polderman KH. Hypothermia and coagulation. Crit Care 2012 June 16;16(Suppl 2):A20-A20.

120. Joshi B, Brady K, Lee J, Easley B, Panigrahi R, Smielewski P, et al. Impaired autoregulation of cerebral blood flow during rewarming from hypothermic cardiopulmonary bypass and its potential association with stroke. Anesth Analg 2010 -2-01;110(2):321-328.

121. Scaravilli V, Bonacina D, Citerio G. Rewarming: Facts and myths from the systemic perspective. Crit Care 2012;16(Suppl 2):A25.

122. Ueda Y, Suehiro E, Wei EP, Kontos HA, Povlishock JT. Uncomplicated rapid posthypothermic rewarming alters cerebrovascular responsiveness. Stroke 2004 Feb 1,;35(2):601-606.

123. Suehiro E, Povlishock JT. Exacerbation of traumatically induced axonal injury by rapid posthypothermic rewarming and attenuation of axonal change by cyclosporin A. J Neurosurg 2001 Mar;94(3):493-498.

124. Nakamura T, Miyamoto O, Yamagami S, Hayashida Y, Itano T, Nagao S. Influence of rewarming conditions after hypothermia in gerbils with transient forebrain ischemia. J Neurosurg 1999 Jul;91(1):114-120.

125. Jiang J, Xu W, Li W, Gao G, Bao Y, Liang Y, et al. Effect of long-term mild hypothermia or short-term mild hypothermia on outcome of patients with severe traumatic brain injury. Journal of Cerebral Blood Flow & Metabolism 2006 Jun;26(6):771-776.

126. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

127. Madhavan K, Benglis DM, Wang MY, Vanni S, Lebwohl N, Green BA, et al. The use of modest systemic hypothermia after iatrogenic spinal cord injury during surgery. Ther Hypothermia Temp Manag 2012 Dec;2(4):183-192.

128. Ahmad FU, Wang MY, Levi AD. Hypothermia for acute spinal cord Injury—A review. World Neurosurgery 2014 Jul;82(1-2):207-214.

129. Dietrich WD, Bramlett HM. Therapeutic hypothermia and targeted temperature management for traumatic brain injury: Experimental and clinical experience. Brain circulation 2017 Oct;3(4):186-198.

130. Andrews PJD, Sinclair HL, Rodriguez A, Harris BA, Battison CG, Rhodes JKJ, et al. Hypothermia for intracranial hypertension after traumatic brain injury. The New England Journal of Medicine 2015 Dec 17,;373(25):2403-2412.

131. Steiner T, Friede T, Aschoff A, Schellinger PD, Schwab S, Hacke W. Effect and feasibility of controlled rewarming after moderate hypothermia in stroke patients with malignant infarction of the middle cerebral artery. Stroke 2001 Dec 01,;32(12):2833-2835.

132. van der Worp, H. Bart, Macleod MR, Bath PMW, Demotes J, Durand-Zaleski I, Gebhardt B, et al. EuroHYP-1: European multicenter, randomized, phase III clinical trial of therapeutic hypothermia plus best medical treatment vs. best medical treatment alone for acute ischemic stroke. International Journal of Stroke 2014 Jul;9(5):642-645.

133. Dohi K, Jimbo H, Ikeda Y, Fujita S, Ohtaki H, Shioda S, et al. Pharmacological brain cooling with indomethacin in acute hemorrhagic stroke: Antiinflammatory cytokines and antioxidative effects. Acta Neurochir Suppl 2006;96:57-60.

134. BI Y, HUAN Y, CAI W, WANG X, LIANG Z, LIU Z, et al. Mild hypothermia in combination with minimally invasive evacuation of hematoma reduces inflammatory damage in patients via the nuclear factor-κB pathway. Experimental and Therapeutic Medicine 2014 Dec;8(6):1717-1722.

135. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

136. Kollmar R, Juettler E, Huttner HB, Dörfler A, Staykov D, Kallmuenzer B, et al. Cooling in intracerebral hemorrhage (CINCH) trial: Protocol of a randomized german-austrian clinical trial. Int J Stroke 2012 Feb;7(2):168-172.

137. Rincon F, Friedman DP, Bell R, Mayer SA, Bray PF. Targeted temperature management after intracerebral hemorrhage (TTM-ICH): Methodology of a prospective randomized clinical trial. Int J Stroke 2014 Jul;9(5):646-651.

138. Sun H, Tang Y, Li L, Guan X, Wang D. Effects of local hypothermia on neuronal cell apoptosis after intracerebral hemorrhage in rats. J Nutr Health Aging 2015 Mar;19(3):291-298.

139. Kawanishi M, Kawai N, Nakamura T, Luo C, Tamiya T, Nagao S. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. J Stroke Cerebrovasc Dis 2008;17(4):187-195.

140. Wei S, Sun J, Li J, Wang L, Hall CL, Dix TA, et al. Acute and delayed protective effects of pharmacologically induced hypothermia in an intracerebral hemorrhage stroke model of mice. Neuroscience 2013;252:489-500.

141. Zhu Y, Liu C, Sun Z. Early combined therapy with pharmacologically induced hypothermia and edaravone exerts neuroprotective effects in a rat model of intracerebral hemorrhage. Cell Biochem Biophys 2015 Nov;73(2):581-587.

142. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

143. Fingas M, Penner M, Silasi G, Colbourne F. Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia. Experimental Neurology 2009;219(1):156-162.

144. Klahr AC, Dietrich K, Dickson CT, Colbourne F. Prolonged localized mild hypothermia does not affect seizure activity after intracerebral hemorrhage in rats. Ther Hypothermia Temp Manag 2016 Mar;6(1):40-47.

145. MacLellan C, Shuaib A, Colbourne F. Failure of delayed and prolonged hypothermia to favorably affect hemorrhagic stroke in rats. Brain Research 2002;958(1):192-200.

146. Sun H, Tang Y, Guan X, Li L, Wang D. Effects of selective hypothermia on blood-brain barrier integrity and tight junction protein expression levels after intracerebral hemorrhage in rats. Biol Chem 2013 Oct;394(10):1317-1324.

147. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

148. Maclellan CL, Grams J, Adams K, Colbourne F. Combined use of a cytoprotectant and rehabilitation therapy after severe intracerebral hemorrhage in rats. Brain Res 2005 Nov 23,;1063(1):40-47.

149. Kawai N, Nakamura T, Nagao S. Effects of brain hypothermia on brain edema formation after intracerebral hemorrhage in rats. Acta Neurochir Suppl 2002;81:233-235.

150. Wagner KR, Beiler S, Beiler C, Kirkman J, Casey K, Robinson T, et al. Delayed profound local brain hypothermia markedly reduces interleukin-1beta gene expression and vasogenic edema development in a porcine model of intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:177-182.

151. Colbourne F, Sutherland GR, Auer RN. An automated system for regulating brain temperature in awake and freely moving rodents. J Neurosci Methods 1996 Aug;67(2):185-190.

152. Proudfoot AT, Simpson D, Dyson EH. Management of acute iron poisoning. Medical toxicology 1986 Mar;1(2):83-100.

153. Gu Y, Hua Y, Keep RF, Morgenstern LB, Xi G. Deferoxamine reduces intracerebral hematoma-induced iron accumulation and neuronal death in piglets. Stroke 2009 Jun 1,;40(6):2241-2243.

154. Nakamura T, Keep RF, Hua Y, Schallert T, Hoff JT, Xi G. Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage. Journal of neurosurgery 2004 Apr;100(4):672-678.

155. Okauchi M, Hua Y, Keep RF, Morgenstern LB, Schallert T, Xi G. Deferoxamine treatment for intracerebral hemorrhage in aged rats: Therapeutic time window and optimal duration. Stroke 2010 Feb 1,;41(2):375-382.

156. Warkentin LM, Auriat AM, Wowk S, Colbourne F. Failure of deferoxamine, an iron chelator, to improve outcome after collagenase-induced intracerebral hemorrhage in rats. Brain Research 2009;1309:95-103.

157. Wu H, Wu T, Xu X, Wang J, Wang J. Iron toxicity in mice with collagenase-induced intracerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2011 May;31(5):1243-1250.

158. Selim M, Yeatts S, Goldstein JN, Gomes J, Greenberg S, Morgenstern LB, et al. Safety and tolerability of deferoxamine mesylate in patients with acute intracerebral hemorrhage. Stroke 2011 Nov;42(11):3067-3074.

159. Selim M, Foster LD, Moy CS, Xi G, Hill MD, Morgenstern LB, et al. Deferoxamine mesylate in patients with intracerebral haemorrhage (i-DEF): A multicentre, randomised, placebo-controlled, double-blind phase 2 trial. The Lancet Neurology 2019 May;18(5):428-438.

160. Horky LL, Pluta RM, Boock RJ, Oldfield EH. Role of ferrous iron chelator 2,2′-dipyridyl in preventing delayed vasospasm in a primate model of subarachnoid hemorrhage. Journal of neurosurgery 1998 Feb;88(2):298-303.

161. Ikeda Y, Ikeda K, Long DM. Comparative study of different iron-chelating agents in cold-induced brain edema. Neurosurgery 1989 Jun;24(6):820-824.

162. Kress GJ, Dineley KE, Reynolds IJ. The relationship between intracellular free iron and cell injury in cultured neurons, astrocytes, and oligodendrocytes. Journal of Neuroscience 2002 Jul 15,;22(14):5848-5855.

163. Nunez MT, Cole ES, Glass J. The reticulocyte plasma membrane pathway of iron uptake as determined by the mechanism of alpha, alpha'-dipyridyl inhibition. J Biol Chem 1983 Jan 25,;258(2):1146-1151.

164. Yu YY, Niu L, Gao L, Zhang GL, Li J, Deng JP, et al. Ferrous chelator 2,2-dipyridyl attenuates cerebral vasospasm after experimental subarachnoid haemorrhage in rabbits. The Journal of International Medical Research 2010 Mar;38(2):583-592.

165. Wu H, Wu T, Li M, Wang J. Efficacy of the lipid-soluble iron chelator 2,2'-dipyridyl against hemorrhagic brain injury. Neurobiol Dis 2012 Jan;45(1):388-394.

Chapter Two

1. van der Worp, H. Bart, Sena ES, Donnan GA, Howells DW, Macleod MR. Hypothermia in animal models of acute ischaemic stroke: A systematic review and meta-analysis. Brain 2007 Dec;130(Pt 12):3063-3074.

2. MacLellan CL, Peeling J, Colbourne F. Cytoprotection strategies for experimental intracerebral hemorrhage (ch 19).

. In: Carpuahoma JR, Mayer SA, Hanley DF, editors. Textbook of intracerebral hemorrhage. New York: Cambridge University Press; 2009.

3. van der Worp, H. Bart, Macleod MR, Kollmar R. Therapeutic hypothermia for acute ischemic stroke: Ready to start large randomized trials? J Cereb Blood Flow Metab 2010 Jun;30(6):1079-1093.

4. Stroke Therapy Academic Industry Roundtable. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 1999 Dec;30(12):2752-2758.

5. O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp, Bart H, Howells DW. 1,026 experimental treatments in acute stroke. Annals of Neurology 2006 Mar;59(3):467-477.

6. Macleod MR, Fisher M, O'Collins V, Sena ES, Dirnagl U, Bath PMW, et al. Good laboratory practice: Preventing introduction of bias at the bench. Stroke 2009 Mar;40(3):50.

7. Lapchak PA, Zhang JH. Resolving the negative data publication dilemma in translational stroke research. Transl Stroke Res 2011 Mar;2(1):1-6.

8. MacLellan CL, Clark DL, Silasi G, Colbourne F. Use of prolonged hypothermia to treat ischemic and hemorrhagic stroke. J Neurotrauma 2009 Mar;26(3):313-323.

9. The Hypothermia after Cardiac Arrest Study Group. Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. The New England Journal of Medicine 2002 Feb 21,;346(8):549-556.

10. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med 2005 Oct 13,;353(15):1574-1584.

11. Lyden PD, Hemmen TM, Grotta J, Rapp K, Raman R. Endovascular therapeutic hypothermia for acute ischemic stroke: ICTuS 2/3 protocol. International Journal of Stroke 2014 Jan;9(1):117-125.

12. van der Worp, H. Bart, Macleod MR, Bath PMW, Demotes J, Durand-Zaleski I, Gebhardt B, et al. EuroHYP-1: European multicenter, randomized, phase III clinical trial of therapeutic hypothermia plus best medical treatment vs. best medical treatment alone for acute ischemic stroke. International Journal of Stroke 2014 Jul;9(5):642-645.

13. Dai D, Wang D, Li K, Mao Y, Zhang L, Duan S, et al. [Effect of local mild hypothermia on expression of aquaporin-4 following intracerebral hemorrhage in rats]. Zhonghua Yi Xue Za Zhi 2006 Apr;86(13):906-910.

14. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

15. Fingas M, Penner M, Silasi G, Colbourne F. Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia. Experimental Neurology 2009;219(1):156-162.

16. Kawanishi M. Effect of hypothermia on brain edema formation following intracerebral hemorrhage in rats. Acta Neurochir Suppl 2003;86:453-456.

17. Kawanishi M, MD, Kawai N, MD, Nakamura T, MD, Luo C, MD, Tamiya T, MD, Nagao S, MD. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. Journal of Stroke and Cerebrovascular Diseases 2008;17(4):187-195.

18. MacLellan C, Shuaib A, Colbourne F. Failure of delayed and prolonged hypothermia to favorably affect hemorrhagic stroke in rats. Brain Research 2002;958(1):192-200.

19. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

20. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

21. Wagner KR, Beiler S, Beiler C, Kirkman J, Casey K, Robinson T, et al. Delayed profound local brain hypothermia markedly reduces interleukin-1beta gene expression and vasogenic edema development in a porcine model of intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:177-182.

22. Wei S, Sun J, Li J, Wang L, Hall CL, Dix TA, et al. Acute and delayed protective effects of pharmacologically induced hypothermia in an intracerebral hemorrhage stroke model of mice. Neuroscience 2013;252:489-500.

23. Feng H, Shi D, Wang D, Xin X, Feng L, Zhang Y, et al. [Effect of local mild hypothermia on treatment of acute intracerebral hemorrhage, a clinical study]. Zhonghua Yi Xue Za Zhi 2002 Dec 10,;82(23):1622-1624.

24. Kollmar R, Staykov D, Dörfler A, Schellinger PD, Schwab S, Bardutzky J. Hypothermia reduces perihemorrhagic edema after intracerebral hemorrhage. Stroke 2010 Aug;41(8):1684-1689.

25. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

26. Rincon F, Friedman DP, Bell R, Mayer SA, Bray PF. Targeted temperature management after intracerebral hemorrhage (TTM-ICH): Methodology of a prospective randomized clinical trial. Int J Stroke 2014 Jul;9(5):646-651.

27. Janny P, Papo I, Chazal J, Colnet G, Barretto LC. Intracranial hypertension and prognosis of spontaneous intracerebral haematomas. A correlative study of 60 patients. Acta Neurochir (Wien) 1982;61(1-3):181-186.

28. Venkatasubramanian C, Mlynash M, Finley-Caulfield A, Eyngorn I, Kalimuthu R, Snider RW, et al. Natural history of perihematomal edema after intracerebral hemorrhage measured by serial magnetic resonance imaging. Stroke 2011 Jan;42(1):73-80.

29. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

30. Arima H, Wang JG, Huang Y, Heeley E, Skulina C, Parsons MW, et al. Significance of perihematomal edema in acute intracerebral hemorrhage: The INTERACT trial. Neurology 2009 Dec;73(23):1963-1968.

31. Hiploylee C, Colbourne F. Intracranial pressure measured in freely moving rats for days after intracerebral hemorrhage. Exp Neurol 2014 May;255:49-55.

32. Kirkman MA, Allan SM, Parry-Jones AR. Experimental intracerebral hemorrhage: Avoiding pitfalls in translational research. J Cereb Blood Flow Metab 2011 Nov;31(11):2135-2151.

33. Babu R, Bagley JH, Di C, Friedman AH, Adamson C. Thrombin and hemin as central factors in the mechanisms of intracerebral hemorrhage-induced secondary brain injury and as potential targets for intervention. Neurosurg Focus 2012 Apr;32(4):E8.

34. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

35. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

36. DeBow S, Colbourne F. Brain temperature measurement and regulation in awake and freely moving rodents. Methods 2003 Jun;30(2):167-171.

37. Clark DL, Colbourne F. A simple method to induce focal brain hypothermia in rats. J Cereb Blood Flow Metab 2007 Jan;27(1):115-122.

38. Clark DL, Penner M, Wowk S, Orellana-Jordan I, Colbourne F. Treatments (12 and 48 h) with systemic and brain-selective hypothermia techniques after permanent focal cerebral ischemia in rat. Exp Neurol 2009 Dec;220(2):391-399.

39. Colbourne F, Corbett D. Delayed and prolonged post-ischemic hypothermia is neuroprotective in the gerbil. Brain Res 1994 Aug 22,;654(2):265-272.

40. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

41. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

42. Schmued LC, Hopkins KJ. Fluoro-jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 2000 Aug;874(2):123-130.

43. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

44. Dietrich WD, Busto R, Alonso O, Globus MY, Ginsberg MD. Intraischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. J Cereb Blood Flow Metab 1993 Jul;13(4):541-549.

45. Caliaperumal J, Brodie S, Ma Y, Colbourne F. Thrombin causes neuronal atrophy and acute but not chronic cell death. Can J Neurol Sci 2014 Nov;41(6):714-720.

46. Silasi G, Colbourne F. Unilateral brain hypothermia as a method to examine efficacy and mechanisms of neuroprotection against global ischemia. Ther Hypothermia Temp Manag 2011;1(2):87-94.

Chapter Three

1. Marion D, Bullock MR. Current and future role of therapeutic hypothermia. Journal of neurotrauma 2009 Mar;26(3):455-467.

2. Yenari MA, Han HS. Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat Rev Neurosci 2012 Feb 22,;13(4):267-278.

3. Goossens J, Hachimi-Idrissi S. Combination of therapeutic hypothermia and other neuroprotective strategies after an ischemic cerebral insult. Curr Neuropharmacol 2014 Sep;12(5):399-412.

4. Feng H, Shi D, Wang D, Xin X, Feng L, Zhang Y, et al. [Effect of local mild hypothermia on treatment of acute intracerebral hemorrhage, a clinical study]. Zhonghua Yi Xue Za Zhi 2002 Dec 10,;82(23):1622-1624.

5. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

6. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

7. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

8. Kawanishi M. Effect of hypothermia on brain edema formation following intracerebral hemorrhage in rats. Acta Neurochir Suppl 2003;86:453-456.

9. Kawanishi M, MD, Kawai N, MD, Nakamura T, MD, Luo C, MD, Tamiya T, MD, Nagao S, MD. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. Journal of Stroke and Cerebrovascular Diseases 2008;17(4):187-195.

10. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

11. Wagner KR, Zuccarello M. Local brain hypothermia for neuroprotection in stroke treatment and aneurysm repair. Neurol Res 2005 Apr;27(3):238-245.

12. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

13. Sun H, Tang Y, Guan X, Li L, Wang D. Effects of selective hypothermia on blood-brain barrier integrity and tight junction protein expression levels after intracerebral hemorrhage in rats. Biol Chem 2013 Oct;394(10):1317-1324.

14. Sun H, Tang Y, Li L, Guan X, Wang D. Effects of local hypothermia on neuronal cell apoptosis after intracerebral hemorrhage in rats. J Nutr Health Aging 2015 Mar;19(3):291-298.

15. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

16. Wowk S, Ma Y, Colbourne F. Mild therapeutic hypothermia does not reduce thrombin-induced brain injury. Ther Hypothermia Temp Manag 2014 Dec;4(4):180-187.

17. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: Secondary brain injury. Stroke 2011 Jun;42(6):1781-1786.

18. Cui H, He H, Yang A, Zhou H, Wang C, Luo J, et al. Efficacy of deferoxamine in animal models of intracerebral hemorrhage: A systematic review and stratified meta-analysis. PLoS One 2015 -5-22;10(5).

19. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

20. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

21. Willmore LJ, Ueda Y. Posttraumatic epilepsy: Hemorrhage, free radicals and the molecular regulation of glutamate. Neurochem Res 2009 Apr;34(4):688-697.

22. Willmore LJ, Ballinger WE, Boggs W, Sypert GW, Rubin JJ. Dendritic alterations in rat isocortex within an iron-induced chronic epileptic focus. Neurosurgery 1980 Aug;7(2):142-146.

23. Colbourne F, Sutherland GR, Auer RN. An automated system for regulating brain temperature in awake and freely moving rodents. J Neurosci Methods 1996 Aug;67(2):185-190.

24. Colbourne F, Li H, Buchan AM. Indefatigable CA1 sector neuroprotection with mild hypothermia induced 6 hours after severe forebrain ischemia in rats. J Cereb Blood Flow Metab 1999 Jul;19(7):742-749.

25. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

26. Auriat AM, Klahr AC, Silasi G, MacLellan CL, Penner M, Clark DL, et al. Prolonged hypothermia in rat: A safety study using brain-selective and systemic treatments. Therapeutic Hypothermia and Temperature Management 2012 Mar 1,;2(1):37-43.

27. John RF, Williamson MR, Dietrich K, Colbourne F. Localized hypothermia aggravates bleeding in the collagenase model of intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2015 Mar 1,;5(1):19-25.

28. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

29. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

Chapter Four

1. Foulkes MA, Wolf PA, Price TR, Mohr JP, Hier DB. The stroke data bank: Design, methods, and baseline characteristics. Stroke 1988 May;19(5):547-554.

2. Sacco S, Marini C, Toni D, Olivieri L, Carolei A. Incidence and 10-year survival of intracerebral hemorrhage in a population-based registry. Stroke 2009 Feb 1,;40(2):394-399.

3. Davis SM, Broderick J, Hennerici M, Brun NC, Diringer MN, Mayer SA, et al. Hematoma growth is a determinant of mortality and poor outcome after intracerebral hemorrhage. Neurology 2006 Apr 25,;66(8):1175-1181.

4. Hackett MJ, DeSouza M, Caine S, Bewer B, Nichol H, Paterson PG, et al. A new method to image heme-fe, total fe, and aggregated protein levels after intracerebral hemorrhage. ACS Chem Neurosci 2015;6(5):761-770.

5. Testai FD, Aiyagari V. Acute hemorrhagic stroke pathophysiology and medical interventions: Blood pressure control, management of anticoagulant-associated brain hemorrhage and general management principles. Neurol Clin 2008 Nov;26(4):963-ix.

6. Wu X, Stezoski J, Safar P, Bauer A, Tuerler A, Schwarz N, et al. Mild hypothermia during hemorrhagic shock in rats improves survival without significant effects on inflammatory responses. Crit Care Med 2003 Jan;31(1):195-202.

7. Howes D, Gray SH, Brooks SC, Boyd JG, Djogovic D, Golan E, et al. Canadian guidelines for the use of targeted temperature management (therapeutic hypothermia) after cardiac arrest: A joint statement from the canadian critical care society (CCCS), canadian neurocritical care society (CNCCS), and the canadian critical care trials group (CCCTG). Resuscitation 2016 Jan;98:48-63.

8. Peliowski-Davidovich A. Hypothermia for newborns with hypoxic ischemic encephalopathy. Paediatrics & child health 2012 Jan;17(1):41-43.

9. Yenari MA, Han HS. Neuroprotective mechanisms of hypothermia in brain ischaemia. Nat Rev Neurosci 2012 Feb 22,;13(4):267-278.

10. Darwazeh R, Yan Y. Mild hypothermia as a treatment for central nervous system injuries: Positive or negative effects. Neural Regen Res 2013 Oct;8(28):2677-2686.

11. Marion D, Bullock MR. Current and future role of therapeutic hypothermia. Journal of neurotrauma 2009 Mar;26(3):455-467.

12. Sherman AL, Wang MY. Hypothermia as a clinical neuroprotectant. Phys Med Rehabil Clin N Am 2014 Aug;25(3):519-529, vii.

13. Lyden PD, Hemmen TM, Grotta J, Rapp K, Raman R. Endovascular therapeutic hypothermia for acute ischemic stroke: ICTuS 2/3 protocol. International Journal of Stroke 2014 Jan;9(1):117-125.

14. Hemmen TM, Raman R, Guluma KZ, Meyer BC, Gomes JA, Cruz-Flores S, et al. Intravenous thrombolysis plus hypothermia for acute treatment of ischemic stroke (ICTuS-L): Final results. Stroke 2010 Oct;41(10):2265-2270.

15. van der Worp, H. Bart, Macleod MR, Bath PMW, Demotes J, Durand-Zaleski I, Gebhardt B, et al. EuroHYP-1: European multicenter, randomized, phase III clinical trial of therapeutic hypothermia plus best medical treatment vs. best medical treatment alone for acute ischemic stroke. International Journal of Stroke 2014 Jul;9(5):642-645.

16. Fingas M, Clark DL, Colbourne F. The effects of selective brain hypothermia on intracerebral hemorrhage in rats. Experimental Neurology 2007;208(2):277-284.

17. Kawanishi M, Kawai N, Nakamura T, Luo C, Tamiya T, Nagao S. Effect of delayed mild brain hypothermia on edema formation after intracerebral hemorrhage in rats. J Stroke Cerebrovasc Dis 2008;17(4):187-195.

18. Liu X, Liu X, Jing L, Jing L, Yang M, Yang M, et al. Enhanced neuroprotection of minimally invasive surgery joint local cooling lavage against ICH-induced inflammation injury and apoptosis in rats. Cell Mol Neurobiol 2016 Jul;36(5):647-655.

19. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

20. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

21. Fingas M, Penner M, Silasi G, Colbourne F. Treatment of intracerebral hemorrhage in rats with 12 h, 3 days and 6 days of selective brain hypothermia. Experimental Neurology 2009;219(1):156-162.

22. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

23. Wu J, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. Brain Res 2002 Oct 25,;953(1-2):45-52.

24. Klahr AC, Dietrich K, Dickson CT, Colbourne F. Prolonged localized mild hypothermia does not affect seizure activity after intracerebral hemorrhage in rats. Ther Hypothermia Temp Manag 2016 Mar;6(1):40-47.

25. John RF, Williamson MR, Dietrich K, Colbourne F. Localized hypothermia aggravates bleeding in the collagenase model of intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2015 Mar 1,;5(1):19-25.

26. Staykov D, Wagner I, Volbers B, Doerfler A, Schwab S, Kollmar R. Mild prolonged hypothermia for large intracerebral hemorrhage. Neurocrit Care 2013 Apr;18(2):178-183.

27. Dohi K, Jimbo H, Ikeda Y, Fujita S, Ohtaki H, Shioda S, et al. Pharmacological brain cooling with indomethacin in acute hemorrhagic stroke: Antiinflammatory cytokines and antioxidative effects. Acta Neurochir Suppl 2006;96:57-60.

28. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

29. Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, et al. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. Stroke 2007 Dec;38(12):3280-3286.

30. Zhao X, Gonzales N, Aronowski J. Pleiotropic role of PPARγ in intracerebral hemorrhage: An intricate system involving Nrf2, RXR, and NF-κB. CNS Neurosci Ther 2015 Apr;21(4):357-366.

31. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: Secondary brain injury. Stroke 2011 Jun;42(6):1781-1786.

32. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

33. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: Reporting in vivo experiments: The ARRIVE guidelines. British Journal of Pharmacology 2010 Aug;160(7):1577-1579.

34. Rosenberg GA, Mun-Bryce S, Wesley M, Kornfeld M. Collagenase-induced intracerebral hemorrhage in rats. Stroke 1990 May;21(5):801-807.

35. Colbourne F, Sutherland GR, Auer RN. An automated system for regulating brain temperature in awake and freely moving rodents. J Neurosci Methods 1996 Aug;67(2):185-190.

36. Klahr AC, Nadeau CA, Colbourne F. Temperature control in rodent neuroprotection studies: Methods and challenges. Ther Hypothermia Temp Manag 2017 Mar;7(1):42-49.

37. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

38. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

39. Zheng W, Nichol H, Liu S, Cheng YN, Haacke EM. Measuring iron in the brain using quantitative susceptibility mapping and X-ray fluorescence imaging. Neuroimage 2013 Sep;78:68-74.

40. Zheng W, Haacke EM, Webb SM, Nichol H. Imaging of stroke: A comparison between X-ray fluorescence and magnetic resonance imaging methods. Magn Reson Imaging 2012;30(10):1416-1423.

41. The microanalysis toolkit: X-ray fluorescence image processing software. Chicago; August 2011.

42. Rebouche CJ, Wilcox CL, Widness JA. Microanalysis of non-heme iron in animal tissues. J Biochem Biophys Methods 2004 Mar 31;58(3):239-251.

43. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

44. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

45. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

46. Choudhri TF, Hoh BL, Solomon RA, Connolly ES, Pinsky DJ. Use of a spectrophotometric hemoglobin assay to objectively quantify intracerebral hemorrhage in mice. Stroke 1997 Nov;28(11):2296-2302.

47. Wowk S, Ma Y, Colbourne F. Therapeutic hypothermia does not mitigate iron-induced injury in rat. Ther Hypothermia Temp Manag 2016 Mar;6(1):23-29.

48. Sheng SP, Lei B, James ML, Lascola CD, Venkatraman TN, Jung JY, et al. Xenon neuroprotection in experimental stroke: Interactions with hypothermia and intracerebral hemorrhage. Anesthesiology 2012 Dec;117(6):1262-1275.

49. Broderick JP, Brott TG, Duldner JE, Tomsick T, Huster G. Volume of intracerebral hemorrhage. A powerful and easy-to-use predictor of 30-day mortality. Stroke 1993 Jul;24(7):987-993.

50. Rost NS, Smith EE, Chang Y, Snider RW, Chanderraj R, Schwab K, et al. Prediction of functional outcome in patients with primary intracerebral hemorrhage: The FUNC score. Stroke 2008 Aug 1,;39(8):2304-2309.

51. Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. Prog Neurobiol 2010 Dec;92(4):463-477.

52. Wu J, Yang S, Hua Y, Liu W, Keep RF, Xi G. Minocycline attenuates brain edema, brain atrophy and neurological deficits after intracerebral hemorrhage. Acta Neurochir Suppl 2010;106:147-150.

53. Xue M, Mikliaeva EI, Casha S, Zygun D, Demchuk A, Yong VW. Improving outcomes of neuroprotection by minocycline: Guides from cell culture and intracerebral hemorrhage in mice. American Journal of Pathology 2010 Mar 1,;176(3):1193-1202.

54. Moxon-Emre I, Schlichter LC. Neutrophil depletion reduces blood-brain barrier breakdown, axon injury, and inflammation after intracerebral hemorrhage. J Neuropathol Exp Neurol 2011 Mar;70(3):218-235.

55. Sansing LH, Harris TH, Kasner SE, Hunter CA, Kariko K. Neutrophil depletion diminishes monocyte infiltration and improves functional outcome after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2011;111:173-178.

56. Andresen M, Gazmuri JT, Marín A, Regueira T, Rovegno M. Therapeutic hypothermia for acute brain injuries. Scand J Trauma Resusc Emerg Med 2015 Jun;23:42.

57. Wagner KR, Beiler S, Beiler C, Kirkman J, Casey K, Robinson T, et al. Delayed profound local brain hypothermia markedly reduces interleukin-1beta gene expression and vasogenic edema development in a porcine model of intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:177-182.

58. Diestel A, Troeller S, Billecke N, Sauer IM, Berger F, Schmitt KRL. Mechanisms of hypothermia-induced cell protection mediated by microglial cells in vitro. Eur J Neurosci 2010 Mar;31(5):779-787.

59. Kawabori M, Hokari M, Zheng Z, Kim JY, Calosing C, Hsieh CL, et al. Triggering receptor expressed on myeloid cells-2 correlates to hypothermic neuroprotection in ischemic stroke. Therapeutic Hypothermia and Temperature Management 2013 Dec 1,;3(4):189-198.

60. Williamson MR, Dietrich K, Hackett MJ, Caine S, Nadeau CA, Aziz JR, et al. Rehabilitation augments hematoma clearance and attenuates oxidative injury and ion dyshomeostasis after brain hemorrhage. Stroke 2017;48(1):195-203.

Chapter Five

1. Sacco S, Marini C, Toni D, Olivieri L, Carolei A. Incidence and 10-year survival of intracerebral hemorrhage in a population-based registry. Stroke 2009 Feb 1,;40(2):394-399.

2. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

3. Rincon F, Mayer SA. Intracerebral hemorrhage: Clinical overview and pathophysiologic concepts. Translational stroke research 2012 Jul;3(Suppl 1):10-24.

4. Selim M. Deferoxamine mesylate: A new hope for intracerebral hemorrhage: From bench to clinical trials. Stroke 2009 Mar 1,;40(3, Supplement 1):S90-S91.

5. Wagner KR, Sharp FR, Ardizzone TD, Lu A, Clark JF. Heme and iron metabolism: Role in cerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2003 Jun;23(6):629-652.

6. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke 2003 Dec 1,;34(12):2964-2969.

7. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

8. Wu H, Wu T, Li M, Wang J. Efficacy of the lipid-soluble iron chelator 2,2'-dipyridyl against hemorrhagic brain injury. Neurobiol Dis 2012 Jan;45(1):388-394.

9. Kell DB. Iron behaving badly: Inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC Med Genomics 2009 Jan 08,;2:2.

10. Kell DB. Towards a unifying, systems biology understanding of large-scale cellular death and destruction caused by poorly liganded iron: Parkinson's, huntington's, alzheimer's, prions, bactericides, chemical toxicology and others as examples. Arch Toxicol 2010 Nov;84(11):825-889.

11. Willmore LJ, Rubin JJ. Formation of malonaldehyde and focal brain edema induced by subpial injection of FeCl2 into rat isocortex. Brain research 1982 Aug 19,;246(1):113-119.

12. Willmore LJ, Ballinger WE, Boggs W, Sypert GW, Rubin JJ. Dendritic alterations in rat isocortex within an iron-induced chronic epileptic focus. Neurosurgery 1980 Aug;7(2):142-146.

13. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

14. Nguyen AP, Huynh HD, Sjovold SB, Colbourne F. Progressive brain damage and alterations in dendritic arborization after collagenase-induced intracerebral hemorrhage in rats. Current neurovascular research 2008 Aug;5(3):171.

15. Okauchi M, Hua Y, Keep RF, Morgenstern LB, Schallert T, Xi G. Deferoxamine treatment for intracerebral hemorrhage in aged rats: Therapeutic time window and optimal duration. Stroke 2010 Feb 1,;41(2):375-382.

16. Wu H, Wu T, Xu X, Wang J, Wang J. Iron toxicity in mice with collagenase-induced intracerebral hemorrhage. Journal of Cerebral Blood Flow & Metabolism 2011 May;31(5):1243-1250.

17. Selim M, Yeatts S, Goldstein JN, Gomes J, Greenberg S, Morgenstern LB, et al. Safety and tolerability of deferoxamine mesylate in patients with acute intracerebral hemorrhage. Stroke 2011 Nov;42(11):3067-3074.

18. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

19. Warkentin LM, Auriat AM, Wowk S, Colbourne F. Failure of deferoxamine, an iron chelator, to improve outcome after collagenase-induced intracerebral hemorrhage in rats. Brain Research 2009;1309:95-103.

20. Kress GJ, Dineley KE, Reynolds IJ. The relationship between intracellular free iron and cell injury in cultured neurons, astrocytes, and oligodendrocytes. Journal of Neuroscience 2002 Jul 15,;22(14):5848-5855.

21. Yu YY, Niu L, Gao L, Zhang GL, Li J, Deng JP, et al. Ferrous chelator 2,2-dipyridyl attenuates cerebral vasospasm after experimental subarachnoid haemorrhage in rabbits. The Journal of International Medical Research 2010 Mar;38(2):583-592.

22. Méthy D, Bertrand N, Prigent-Tessier A, Mossiat C, Stanimirovic D, Beley A, et al. Beneficial effect of dipyridyl, a liposoluble iron chelator against focal cerebral ischemia: In vivo and in vitro evidence of protection of cerebral endothelial cells. Brain Research 2007;1193:136-142.

23. Del Bigio MR, Yan HJ, Buist R, Peeling J. Experimental intracerebral hemorrhage in rats. magnetic resonance imaging and histopathological correlates. Stroke 1996 Dec;27(12):2312-2320.

24. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

25. Rosenberg GA, Mun-Bryce S, Wesley M, Kornfeld M. Collagenase-induced intracerebral hemorrhage in rats. Stroke 1990 May;21(5):801-807.

26. MacLellan CL, Auriat AM, McGie SC, Yan RHY, Huynh HD, De Butte MF, et al. Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies. J Cereb Blood Flow Metab 2006 Aug;26(8):1031-1042.

27. Schallert T. Behavioral tests for preclinical intervention assessment. NeuroRx 2006 Oct;3(4):497-504.

28. Metz GA, Whishaw IQ. Cortical and subcortical lesions impair skilled walking in the ladder rung walking test: A new task to evaluate fore- and hindlimb stepping, placing, and co-ordination. J Neurosci Methods 2002 Apr;115(2):169-179.

29. Rebouche CJ, Wilcox CL, Widness JA. Microanalysis of non-heme iron in animal tissues. J Biochem Biophys Methods 2004 Mar 31;58(3):239-251.

30. MacLellan CL, Davies LM, Fingas MS, Colbourne F. The influence of hypothermia on outcome after intracerebral hemorrhage in rats. Stroke 2006 May 1,;37(5):1266-1270.

31. Schmued LC, Hopkins KJ. Fluoro-jade B: A high affinity fluorescent marker for the localization of neuronal degeneration. Brain Res 2000 Aug;874(2):123-130.

32. Pedersen BK. The diseasome of physical inactivity--and the role of myokines in muscle--fat cross talk. J Physiol (Lond) 2009 Dec 01,;587(Pt 23):5559-5568.

33. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

34. Pedersen BK, Pedersen M, Krabbe KS, Bruunsgaard H, Matthews VB, Febbraio MA. Role of exercise-induced brain-derived neurotrophic factor production in the regulation of energy homeostasis in mammals. Exp Physiol 2009 Dec;94(12):1153-1160.

35. Wu G, Xi G, Hua Y, Sagher O. T2* magnetic resonance imaging sequences reflect brain tissue iron deposition following intracerebral hemorrhage. Transl Stroke Res 2010 Mar 01,;1(1):31-34.

36. Lyden PD, Shuaib A, Lees KR, Davalos A, Davis SM, Diener H, et al. Safety and tolerability of NXY-059 for acute intracerebral hemorrhage: The CHANT trial. Stroke 2007 Aug 1,;38(8):2262-2269.

37. Fitch CA, Song Y, Levenson CW. Developmental regulation of hepatic ceruloplasmin mRNA and serum activity by exogenous thyroxine and dexamethasone. Proc Soc Exp Biol Med 1999 May;221(1):27-31.

38. Zhao X, Sun G, Zhang J, Strong R, Dash PK, Kan YW, et al. Transcription factor Nrf2 protects the brain from damage produced by intracerebral hemorrhage. Stroke 2007 Dec;38(12):3280-3286.

39. Zhao X, Song S, Sun G, Strong R, Zhang J, Grotta JC, et al. Neuroprotective role of haptoglobin after intracerebral hemorrhage. J Neurosci 2009 Dec 16,;29(50):15819-15827.

40. Lapchak PA, Zhang JH. Resolving the negative data publication dilemma in translational stroke research. Transl Stroke Res 2011 Mar;2(1):1-6.

Chapter Six

1. MacLellan CL, Clark DL, Silasi G, Colbourne F. Use of prolonged hypothermia to treat ischemic and hemorrhagic stroke. J Neurotrauma 2009 Mar;26(3):313-323.

2. Melmed KR, Lyden PD. Meta-analysis of pre-clinical trials of therapeutic hypothermia for intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2017 Sep;7(3):141-146.

3. Wowk S, Ma Y, Colbourne F. Mild therapeutic hypothermia does not reduce thrombin-induced brain injury. Ther Hypothermia Temp Manag 2014 Dec;4(4):180-187.

4. Wowk S, Ma Y, Colbourne F. Therapeutic hypothermia does not mitigate iron-induced injury in rat. Ther Hypothermia Temp Manag 2016 Mar;6(1):23-29.

5. Caliaperumal J, Brodie S, Ma Y, Colbourne F. Thrombin causes neuronal atrophy and acute but not chronic cell death. Can J Neurol Sci 2014 Nov;41(6):714-720.

6. Keep RF, Dr, Hua Y, MD, Xi G, MD. Intracerebral haemorrhage: Mechanisms of injury and therapeutic targets. Lancet Neurology, The 2012;11(8):720-731.

7. Lee KR, Kawai N, Kim S, Sagher O, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: Effects of thrombin on cerebral blood flow, blood-brain barrier permeability, and cell survival in a rat model. Journal of neurosurgery 1997 Feb;86(2):272.

8. Lee KR, Drury I, Vitarbo E, Hoff JT. Seizures induced by intracerebral injection of thrombin: A model of intracerebral hemorrhage. Journal of neurosurgery 1997 Jul;87(1):73-78.

9. Wu J, Hua Y, Keep RF, Nakamura T, Hoff JT, Xi G. Iron and iron-handling proteins in the brain after intracerebral hemorrhage. Stroke 2003 Dec 1,;34(12):2964-2969.

10. Wu G, Xi G, Hua Y, Sagher O. T2* magnetic resonance imaging sequences reflect brain tissue iron deposition following intracerebral hemorrhage. Transl Stroke Res 2010 Mar 01,;1(1):31-34.

11. Caliaperumal J, Ma Y, Colbourne F. Intraparenchymal ferrous iron infusion causes neuronal atrophy, cell death and progressive tissue loss: Implications for intracerebral hemorrhage. Experimental neurology 2012;237(2):363-369.

12. Kawai N, Kawanishi M, Okauchi M, Nagao S. Effects of hypothermia on thrombin-induced brain edema formation. Brain Res 2001 Mar 23,;895(1-2):50-58.

13. GAO D, DING F, LEI G, LUAN G, ZHANG S, LI K, et al. Effects of focal mild hypothermia on thrombin-induced brain edema formation and the expression of protease activated receptor-1, matrix metalloproteinase-9 and aquaporin 4 in rats. Molecular Medicine Reports 2015 Apr;11(4):3009-3014.

14. MacLellan CL, Paquette R, Colbourne F. A critical appraisal of experimental intracerebral hemorrhage research. J Cereb Blood Flow Metab 2012 Apr;32(4):612-627.

15. Yao Z, You C, He M. Effect and feasibility of therapeutic hypothermia in patients with hemorrhagic stroke: A Systematic review and meta-analysis. World Neurosurgery 2018 Mar;111:404-412.e2.

16. Lyden PD, Lamb J, Kothari S, Toossi S, Boitano P, Rajput PS. Differential effects of hypothermia on neurovascular unit determine protective or toxic results: Toward optimized therapeutic hypothermia. Journal of Cerebral Blood Flow & Metabolism 2019 Sep;39(9):1693-1709.

17. Wan J, Ren H, Wang J. Iron toxicity, lipid peroxidation and ferroptosis after intracerebral haemorrhage. Stroke and vascular neurology 2019 Jul;4(2):93-95.

18. Nakamura T, Keep RF, Hua Y, Nagao S, Hoff JT, Xi G. Iron-induced oxidative brain injury after experimental intracerebral hemorrhage. Acta Neurochir Suppl 2006;96:194-198.

19. Auriat AM, Silasi G, Wei Z, Paquette R, Paterson P, Nichol H, et al. Ferric iron chelation lowers brain iron levels after intracerebral hemorrhage in rats but does not improve outcome. Exp Neurol 2012 Mar;234(1):136-143.

20. Warkentin LM, Auriat AM, Wowk S, Colbourne F. Failure of deferoxamine, an iron chelator, to improve outcome after collagenase-induced intracerebral hemorrhage in rats. Brain Research 2009;1309:95-103.

21. Bridges KR, Cudkowicz A. Effect of iron chelators on the transferrin receptor in K562 cells. The Journal of Biological Chemistry 1984 November;259(21):12970-12977.

22. Caliaperumal J, Wowk S, Jones S, Ma Y, Colbourne F. Bipyridine, an iron chelator, does not lessen intracerebral iron-induced damage or improve outcome after intracerebral hemorrhagic stroke in rats. Transl Stroke Res 2013 Dec;4(6):719-728.

23. Groce DF, Kimbrough RD. Acute and subacute toxicity in sherman strain rats exposed to 4,4'- and 2,2'-dipyridyl. Journal of Toxicology and Environmental Health 1982 Sep 1,;10(3):363-372.

24. Wu H, Wu T, Li M, Wang J. Efficacy of the lipid-soluble iron chelator 2,2'-dipyridyl against hemorrhagic brain injury. Neurobiol Dis 2012 Jan;45(1):388-394.

25. O'Collins VE, Macleod MR, Cox SF, Van Raay L, Aleksoska E, Donnan GA, et al. Preclinical drug evaluation for combination therapy in acute stroke using systematic review, meta-analysis, and subsequent experimental testing. J Cereb Blood Flow Metab 2011 Mar;31(3):962-975.

26. MacLellan CL, Girgis J, Colbourne F. Delayed onset of prolonged hypothermia improves outcome after intracerebral hemorrhage in rats. J Cereb Blood Flow Metab 2004 Apr;24(4):432-440.

27. John RF, Williamson MR, Dietrich K, Colbourne F. Localized hypothermia aggravates bleeding in the collagenase model of intracerebral hemorrhage. Therapeutic Hypothermia and Temperature Management 2015 Mar 1,;5(1):19-25.

28. Berger C, Xia F, Köhrmann M, Schwab S. Hypothermia in acute stroke--slow versus fast rewarming an experimental study in rats. Exp Neurol 2007 Mar;204(1):131-137.

29. Steiner T, Friede T, Aschoff A, Schellinger PD, Schwab S, Hacke W. Effect and feasibility of controlled rewarming after moderate hypothermia in stroke patients with malignant infarction of the middle cerebral artery. Stroke 2001 Dec 1,;32(12):2833-2835.

30. John RF, Colbourne F. Delayed localized hypothermia reduces intracranial pressure following collagenase-induced intracerebral hemorrhage in rat. Brain Res 2016 Feb 15,;1633:27-36.

31. Clark DL, Penner M, Orellana-Jordan IM, Colbourne F. Comparison of 12, 24 and 48 h of systemic hypothermia on outcome after permanent focal ischemia in rat. Exp Neurol 2008 Aug;212(2):386-392.

32. Nakamura T, Miyamoto O, Yamagami S, Hayashida Y, Itano T, Nagao S. Influence of rewarming conditions after hypothermia in gerbils with transient forebrain ischemia. J Neurosurg 1999 Jul;91(1):114-120.

33. Ueda Y, Suehiro E, Wei EP, Kontos HA, Povlishock JT. Uncomplicated rapid posthypothermic rewarming alters cerebrovascular responsiveness. Stroke 2004 Feb 1,;35(2):601-606.

34. MacLellan CL, Silasi G, Poon CC, Edmundson CL, Buist R, Peeling J, et al. Intracerebral hemorrhage models in rat: Comparing collagenase to blood infusion. J Cereb Blood Flow Metab 2008 Mar;28(3):516-525.

35. Nadeau C, Nadeau C, Dietrich K, Dietrich K, Wilkinson C, Wilkinson C, et al. Prolonged blood-brain barrier injury occurs after experimental intracerebral hemorrhage and is not acutely associated with additional bleeding. Transl Stroke Res 2019 Jun 15,;10(3):287-297.

36. Su X, Zheng K, Ma Q, Huang J, He X, Chen G, et al. Effect of local mild hypothermia on regional cerebral blood flow in patients with acute intracerebral hemorrhage assessed by 99mTc-ECD SPECT imaging. J Xray Sci Technol 2015;23(1):101-109.

37. Begley C, Ioannidis J. Reproducibility in science: Improving the standard for basic and preclinical research. Circulation Research 2015 Jan 2,;116(1):116-126.

38. Macleod MR, Fisher M, O'Collins V, Sena ES, Dirnagl U, Bath PMW, et al. Good laboratory practice: Preventing introduction of bias at the bench. Stroke 2009 Mar;40(3):50.

39. Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, et al. Update of the stroke therapy academic industry roundtable preclinical recommendations. Stroke 2009 Jun;40(6):2244-2250.

40. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: Reporting in vivo experiments: The ARRIVE guidelines. British Journal of Pharmacology 2010 Aug;160(7):1577-1579.

41. Stroke Therapy Academic Industry Roundtable. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. Stroke 1999 Dec;30(12):2752-2758.

42. Schlattmann P, Dirnagl U. Statistics in experimental cerebrovascular research-comparison of two groups with a continuous outcome variable. Journal of Cerebral Blood Flow & Metabolism 2010 Mar;30(3):474-479.

43. Roquer J, Rodríguez-Campello A, Jiménez-Conde J, Cuadrado-Godia E, Giralt-Steinhauer E, Vivanco Hidalgo RM, et al. Sex-related differences in primary intracerebral hemorrhage. Neurology 2016 Jul 19,;87(3):257-262.

44. Selim M, Foster LD, Moy CS, Xi G, Hill MD, Morgenstern LB, et al. Deferoxamine mesylate in patients with intracerebral haemorrhage (i-DEF): A multicentre, randomised, placebo-controlled, double-blind phase 2 trial. The Lancet Neurology 2019 May;18(5):428-438.

45. Schmidt-Pogoda A, Bonberg N, Koecke MHM, Strecker J, Wellmann J, Bruckmann N, et al. Why most acute stroke studies are positive in animals but not in patients: A systematic comparison of preclinical, early phase, and phase 3 clinical trials of neuroprotective agents. Ann Neurol 2020 Jan;87(1):40-51.

46. Liu L, Wang Y, Meng X, Li N, Tan Y, Nie X, et al. Tranexamic acid for acute intracerebral hemorrhage growth predicted by spot sign trial: Rationale and design. International Journal of Stroke 2017 Apr;12(3):326-31.

47. Amer AR, Oorschot DE. Xenon combined with hypothermia in perinatal hypoxic-ischemic encephalopathy: A noble gas, a noble mission. Pediatr Neurol 2018;84:5-10.

48. Azzopardi D, Robertson NJ, Bainbridge A, Cady E, Charles-Edwards G, Deierl A, et al. Moderate hypothermia within 6 h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-xe): A proof-of-concept, open-label, randomised controlled trial. Lancet Neurol 2016 -2;15(2):145-153.

49. Mayer SA, Brun NC, Begtrup K, Broderick J, Davis S, Diringer MN, et al. Efficacy and safety of recombinant activated factor VII for acute intracerebral hemorrhage. N Engl J Med 2008 May 15,;358(20):2127-2137.

50. Gladstone DJ, Aviv RI, Demchuk AM, Hill MD, Thorpe KE, Khoury JC, et al. Effect of recombinant activated coagulation factor VII on hemorrhage expansion among patients with spot sign-positive acute intracerebral hemorrhage: The SPOTLIGHT and STOP-IT randomized clinical trials. JAMA neurology 2019 Aug 19,;76(12):1493.

51. Davis SM, Broderick J, Hennerici M, Brun NC, Diringer MN, Mayer SA, et al. Hematoma growth is a determinant of mortality and poor outcome after intracerebral hemorrhage. Neurology 2006 Apr 25,;66(8):1175-1181.

52. Lyden P, Hemmen T, Grotta J, Rapp K, Ernstrom K, Rzesiewicz T, et al. Results of the ICTuS 2 trial (intravascular cooling in the treatment of stroke 2). Stroke 2016;47(12):2888-2895.

53. van der Worp, H Bart, Macleod MR, Bath PM, Bathula R, Christensen H, Colam B, et al. Therapeutic hypothermia for acute ischaemic stroke. results of a european multicentre, randomised, phase III clinical trial. European Stroke Journal 2019 Sep;4(3):254-262.

54. Kollmar R, Juettler E, Huttner HB, Dörfler A, Staykov D, Kallmuenzer B, et al. Cooling in intracerebral hemorrhage (CINCH) trial: Protocol of a randomized german-austrian clinical trial. Int J Stroke 2012 Feb;7(2):168-172.

55. Rincon F, Friedman DP, Bell R, Mayer SA, Bray PF. Targeted temperature management after intracerebral hemorrhage (TTM-ICH): Methodology of a prospective randomized clinical trial. Int J Stroke 2014 Jul;9(5):646-651.

56. Gioia L, Klahr A, Kate M, Buck B, Dowlatshahi D, Jeerakathil T, et al. The intracerebral hemorrhage acutely decreasing arterial pressure trial II (ICH ADAPT II) protocol. BMC neurology 2017 May 19,;17(1):100.

57. Okuda Y, Nakata T. Effect of intensive rehabilitation on improvement of activity of daily living after intracerebral hemorrhage: A retrospective observational study. International Journal of Rehabilitation Research 2019 Oct 29,:1.

58. Paolucci S, Antonucci G, Grasso MG, Bragoni M, Coiro P, De Angelis D, et al. Functional outcome of ischemic and hemorrhagic stroke patients after inpatient rehabilitation: A matched comparison. Stroke 2003 Dec;34(12):2861-2865.

59. Auriat AM, Wowk S, Colbourne F. Rehabilitation after intracerebral hemorrhage in rats improves recovery with enhanced dendritic complexity but no effect on cell proliferation. Behav Brain Res 2010 Dec 06,;214(1):42-47.

60. Caliaperumal J, Colbourne F. Rehabilitation improves behavioral recovery and lessens cell death without affecting iron, ferritin, transferrin, or inflammation after intracerebral hemorrhage in rats. Neurorehabil Neural Repair 2014 May;28(4):395-404.

61. Williamson MR, Dietrich K, Hackett MJ, Caine S, Nadeau CA, Aziz JR, et al. Rehabilitation augments hematoma clearance and attenuates oxidative injury and ion dyshomeostasis after brain hemorrhage. Stroke 2017;48(1):195-203.