University of Alberta

Cytoprotection for Intracerebral Hemorrhagic Stroke in Rats

by

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Abstract

Intracerebral hemorrhage (ICH) is a devastating stroke with no proven clinical therapy. Hypothermia persistently reduces brain injury and impairment in rodent models of global and focal ischemia, and successfully treats cardiac arrest and massive hemispheric stroke. However, the therapeutic potential of hypothermia has not been assessed for ICH.

In chapter 2, we evaluated hypothermia starting 1 hour after ICH created by striatal bacterial collagenase infusion. This treatment, which is most efficacious in ischemia, provided no benefit for ICH. We hypothesized that early hypothermia causes deleterious side effects (e.g., hypertension) that exacerbate ongoing bleeding that occurs in this model. Therefore, delayed treatments might provide greater benefit. In chapter 3, early hypothermia elevated blood pressure and exacerbated bleeding, whereas hypothermia delayed for 12 hours after ICH provided persistent histological and functional benefit. We predicted that early hypothermia would be more efficacious after blood injection, because ongoing bleeding does not occur in this model, and assessed this in chapter 4. Hypothermia reduced secondary consequences of ICH but provided limited functional and histological benefit. Although hypothermia provided some benefit for ICH, we do not recommend its clinical application until side effects are identified, treatment is improved (e.g., optimal duration or use with adjunct therapy), and lasting benefit is demonstrated in multiple models.

In chapter 5, we elevated body temperature following collagenase-induced ICH to investigate the effects of hyperthermia. Contrary to the ischemia literature, mild to

moderate hyperthermia did not worsen outcome. We nonetheless recommend that hyperthermia be avoided in ICH patients.

Finally, in chapters 6 and 7 we tracked recovery in the collagenase and whole blood ICH models using a battery of tests. The goal was to identify tests sensitive to long-term deficits in each model. If implemented, our findings should improve the quality of functional assessment in experimental ICH studies.

In summary, we comprehensively evaluated efficacy and mechanisms of prolonged hypothermia treatment in two rodent ICH models. Although we obtained some benefit in both models, hypothermia must be improved before it is used clinically. Our studies highlight some limitations of hypothermia for ICH, and of experimental ICH studies in general.

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Dedicated to my parents, John and Lorie MacLellan.

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List of Abbreviations:

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BBB	blood-brain barrier
BP	blood pressure
CBF	cerebral blood flow
COX	cyclooxygenase
DNA	deoxyribonucleic acid
h	hour
ICH	intracerebral hemorrhage
IL-1β	interleukin 1-beta
MCAO	middle cerebral artery occlusion
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate)
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NBQX	1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide
NDS	neurological deficit score
NMDA	N-methyl D-aspartate
PET	positron emission tomography
RBC	red blood cell
rFVIIa	recombinant activating factor VII
TNF-α	tumor necrosis factor-alpha
tPA	tissue plasminogen activator

Chapter 1: Introduction

1-1 Classification of Stroke

Stroke is the leading cause of long – term disability and fourth leading cause of death in Canada (Heart and Stroke Foundation of Canada, 2006). Cerebral ischemia represents about 80 % of all strokes and results from a sudden interruption in blood flow to the brain. This may be global, in which the entire brain is affected, such as occurs during cardiac arrest. Alternatively, focal ischemia results from the occlusion of an artery (most commonly the middle cerebral artery), causing an impairment of blood flow to a particular region of the brain. Currently, there are few treatment options for acute stroke. Restoration of blood flow may be successfully achieved with the thrombolytic agent, tissue plasminogen activator (tPA)[1]. However, its use is limited to a small percentage of stroke patients that can be treated within three hours of symptom onset, as greater delays to treatment increase the risk of hemorrhaging[2, 3]. While some recovery of function spontaneously occurs in the weeks or months following a stroke, many patients suffer from lasting disabilities including hemiparesis, aphasia, and cognitive impairments[4].

Hemorrhagic stroke accounts for approximately 20 % of strokes and results from the rupture of a cerebral artery. Depending on the location of the ruptured artery, blood either fills the subarachnoid space surrounding the brain (subarachnoid hemorrhage) or is released into the brain (intracerebral hemorrhage). Hemorrhagic stroke is far more deadly than ischemic stroke, and the majority of deaths occur by the end of the second day. Early death is due to mechanical damage to the brain from the hemorrhage itself, as well as brain herniation and secondary brain stem hemorrhage due to mass effect and substantial elevations in intracranial pressure.

1-2 Intracerebral Hemorrhagic Stroke

Intracerebral hemorrhage (ICH) accounts for ~ 15 % of all strokes and the number of affected individuals is expected to double with an aging population[5, 6]. An ICH is one of the deadliest types of stroke (~ 40 % mortality by one month) and survivors are left with severe and lasting disabilities[7, 8]. Outcome is determined primarily by the location and size of the hemorrhage, and by the initial level of consciousness as determined by the Glasgow Coma Scale[9]. Other independent predictors of poor outcome include the presence of diabetes mellitus and hyperglycemia[10].

Primary ICH (~ 80 % of cases) results from the spontaneous rupture of small arteries originating from the basilar, anterior, middle, or posterior cerebral arteries, usually as a result of damage from chronic hypertension and/or amyloid angiopathy. Most bleeding occurs at the bifurcation of affected arteries, where the media and smooth muscle has substantially degenerated[11]. Thus, ICH most commonly occurs in the basal ganglia (40 %), thalamus (10 %), cerebellum (15 %), or pons (5 %)[7]. Lobar ICH accounts for approximately 30 % of cases. Secondary ICH (~ 20 % of cases) results from vascular abnormalities (e.g., aneurysm, arteriovenous malformation), coagulation defects, trauma, or bleeding into tumors. In addition, hemorrhagic transformation[12] or tPA induced hemorrhaging[13] follows occlusive stroke in approximately 30 % of patients[12]. A hemorrhage in the caudate is often accompanied by intraventricular blood and hydrocephalus, and patients present with confusion, memory loss, contralateral hemiparesis, and gaze paresis. Patients with putaminal hemorrhages present with hemiparesis, hemisensory loss, and occasionally neglect or dysphasia[9].

The deleterious effects of an ICH are broadly divided into two categories: primary and secondary injury. Primary injury refers to immediate effects such as hematoma growth and elevated intracranial pressure. This can lead to brain herniation and death if left untreated. Secondary degenerative events include edema, increased inflammation, peri-hematoma ischemia, and disrupted blood brain barrier (BBB), which may occur soon after ICH and for weeks afterwards[14].

1-3 Risk Factors for ICH and Medical Management

Age is the most important non-modifiable risk factor for ICH, and the incidence of ICH increases exponentially with increasing age. This is likely due to increasing prevalence of hypertension, cerebrovascular amyloid deposition, and normal wear and tear of old blood vessels[7]. Hypertension is the most prevalent and important modifiable risk factor for ICH[15-17] and is present in approximately 60 % of cases[18]. Even borderline systolic hypertension (> 125 mmHg) increases the risk of ICH[17]. ICH originating in the thalamus, basal ganglia, pons, or cerebellum has been associated with vasculopathy of the small penetrating arteries that is strongly linked to hypertension[19]. Hypertension may lead to ICH especially in these areas because walls of the lenticulostriate and paramedian vessels that supply these regions are thinner than similar-sized cortical vessels. Furthermore, these vessels originate directly from main trunk vessels, and are therefore subject to higher intravascular pressures (than similar-sized cortical vessels)[9]. Treatment and control of hypertension appears to reduce the occurrence of intracerebral hemorrhage[20].

Cerebral amyloid angiopathy is characterized by β-amyloid protein build-up on blood vessel walls, causing them to crack and leak, and causes about 15 % of all

intracerebral hemorrhages. Notably, cerebral amyloid angiopathy is a leading cause of ICH in normotensive elderly patients[21]. Other risk factors for ICH include gender (more likely to occur in males), Asian and African American race,[14] coagulation disorders,[7] use of anticoagulants,[22] high serum cholesterol,[23] and alcohol consumption[24]. Finally, the use of illicit drugs (e.g., cocaine and amphetamine) is a major cause of ICH in young adults[25].

As in ischemic stroke, prevention of ICH is preferable to reducing brain injury once an ICH has occurred. The high morbidity, mortality, and economic burden of ICH necessitate the development of therapies to improve outcome. However, management of ICH remains controversial because of the lack of proven medical or surgical therapies. Clinical targets include prevention (e.g., controlling blood pressure[26]), minimizing hematoma expansion,[27, 28] surgery[29] (e.g., aspiration of hematoma), limiting the development of life-threatening consequences of ICH (e.g., edema, elevated intracranial pressure[14]), and using rehabilitation strategies to promote recovery of function[8].

1-4 Pathophysiology of ICH

A surge in studies examining the pathophysiology of ICH has garnered interest and anticipation that an effective treatment is on the horizon. The development of effective therapies depends upon an understanding of the pathophysiology of ICH. The pathology of ICH is similar in humans and other animals, and much of what is known has been acquired though experimental (e.g., rodent, primate, cat) ICH studies. Thus, the pathophysiology of *human* ICH is described below, but is illustrated or supported by many examples from *rodent* studies. While much progress has occurred, ICH remains a complex problem that is incompletely understood.

1-4a Hematoma Enlargement

Until recently, ICH was thought to be a monophasic event, in which bleeding was completed within minutes of onset[30]. Several recent studies, however, report an increased volume of parenchymal hemorrhage on repeated CT scans in 20 – 36 % of patients, leading to poor outcome[31-34]. Hematoma expansion has been attributed to ongoing bleeding from the primary source of ICH, and to mechanical disruption of surrounding vessels. Brain injury related to the hematoma mass results from mechanical force, especially if the hematoma is large. Further enlargement of the hematoma contributes to midline shift and accelerates neurological deterioration[35].

1-4b Edema Formation

The role of edema in the pathology of ICH has received considerable attention. Edema elevates intracranial pressure and causes brain herniation, thereby contributing to mortality[36]. However, its contribution to neurological deficits, and therefore its promise as a putative therapeutic target, remains unclear and must be investigated further[37]. Vasogenic edema develops within 3 hours (h) and peaks between 10-20 days after ICH[38, 39]. There are several phases of edema formation after ICH. In the very early phase (e.g., within the first few hours), there is hydrostatic pressure and clot retraction with movement of serum (which includes osmotically active serum proteins) from the clot into the surrounding tissue[40]. In the second phase, which occurs during the first several days after ICH, components of the coagulation cascade (e.g., clotting factors) and thrombin production induce edema formation[41]. Although thrombin is an essential part of the coagulation cascade, a high concentration of thrombin in the brain induces cell death (e.g., via neurotoxicity[42]), promotes edema formation[43], causes infiltration of inflammatory cells[44], and damages the BBB[45]. Indeed, the breakdown of the BBB, which

regulates the volume of the brain by preventing convective flow of water from the blood, occurs 8-12 h after ICH and also contributes to vasogenic edema formation[46]. The third phase occurs days to weeks after ICH[47] and is associated with erythrocyte lysis and clot resolution. Experimental studies show that the contents of erythrocytes are toxic and lead to edema formation, BBB disruption, and DNA injury (via oxygen free radicals) in rats. Notably, hemoglobin and hemoglobin breakdown products (including iron, carbon monoxide, and biliverdin) contribute to brain injury after ICH[46]. For example, hemoglobin caused edema and brain damage when infused into the brain of rats[48]. Inhibition of heme-oxygenase, which degrades heme, reduces this brain injury[49-51]. Furthermore, intracerebral infusion of iron also caused brain injury in rats[52], which was reduced by the iron-chelator, deferoxamine[53]. These studies suggest that hemoglobin and its breakdown products are toxic and play an important role in brain injury after ICH.

1-4c Cerebral Ischemia

The outward pressure of a hematoma should theoretically compress surrounding tissue, including the vasculature. If so, peri-hematoma ischemia might occur. However, studies in animals and humans are contradictory and have not confirmed this. In humans, a zone of reduced cerebral blood flow (CBF) around the hematoma was reported soon after ICH[54] using positron emission tomography (PET)[55] and single photon emission contrast tomography (SPECT)[56]. Zazulia and colleagues[55] found a reduction in cerebral metabolic rate of oxygen in addition to reduced CBF surrounding the hematoma, indicating that there is a zone of hypoperfusion around the hematoma *without* ischemia. The lack of ischemia in this region was again confirmed using diffusion weighted MRI in

this region[57], and it was hypothesized that the area of decreased CBF might be due to clot retraction and resultant serum accumulation in peri-hematoma region[58].

Experimental studies in rats demonstrated a small and transient reduction in CBF adjacent to the hematoma[59]. Although these findings were not replicated in other animal models of ICH (e.g., in the dog[60]), it is possible that ischemia could occur if the hematoma is immense and intracranial pressure is substantially elevated[46].

1-4d Inflammation

Infiltration of inflammatory cells (e.g., neutrophils) occurs soon after ICH and peaks several days later. The progression of the inflammatory response is similar in humans and other animals (e.g., rats), however, the majority of our knowledge comes from experimental ICH studies. In rodent models of ICH for instance, cytokines are produced within minutes of the ICH. Neutrophil infiltration begins several hours after ICH and peaks within two days, followed by activation and infiltration of macrophages between three and five days, and glial cells over at least one month[61, 62]. Rodent ICH studies suggest that inflammation exacerbates ICH, and it is therefore a potential target for therapy. This is supported by the finding that several anti-inflammatory agents, such as the cyclooxygenase-2 (COX-2) inhibitor celecoxib[63], and tuftstin fragment 1-3[64] decrease inflammation, edema and neurological deficits after ICH in rodents. Further study into the role of other components of inflammation, such as matrix metalloproteinases (MMPs), is warranted. An ICH activates the complement cascade, but its role in ICH injury is unclear. For example, some components of the complement system increase injury via formation of membrane attack complexes that degrade cell membranes and cause cell lysis, whereas others may be beneficial[65].

Although we assume a pathological role for inflammation, one should not lose sight of the necessity of cytokines and inflammation. For example, microglia may contribute to ICH injury, but they also participate in limiting and clearing the hematoma[64]. Acute elevations in tumor necrosis factor-alpha (TNF-α) appear to be harmful and if so, should be mitigated, but basal levels of TNF-α are essential for normal neuronal and glial development and survival [66]. Furthermore, TNF-α is neuroprotective during ischemic stress[67]. In addition, there is increasing evidence that microglia contribute to regeneration after brain injury through the release of cytokines, growth factors, and extracellular matrix molecules [68, 69]. Thus, prior to clinical investigation of potent and/or broad-spectrum anti-inflammatory agents for ICH, further research is needed to increase our understanding of the positive and negative roles of the inflammatory response.

1-4e Cell Death

Neuronal death in the tissue in and around the hematoma is primarily necrotic[70], and results from mechanical compression and chemical toxicity from components released from the blood clot[46]. Although apoptosis occurs in tissue adjacent to the hematoma[71, 72], its role in ICH injury needs further clarification. Specifically, studies must identify the type of cell (e.g., glial cells or neurons) undergoing apoptotic cell death. Furthermore, relying on TUNEL staining is inadequate as it is difficult to distinguish between apoptotic and necrotic cells. Electron microscopy would confirm the mode of cell death, as would assessment of mitochondrial function or the use of specific markers for caspases. Cell death occurs over weeks to months following ICH[73, 74], and causes brain atrophy, as evidenced by a decrease in the size of the striatum and ipsilateral

ventriculomegaly[75]. This ongoing cell death / atrophy represents a target for intervention. Therefore, it would be valuable to determine the mechanisms of atrophy, such as transneuronal degeneration or dendritic thinning.

1-5 Therapeutic Approaches For ICH

To date, there is no effective therapy for ICH. Tissue encompassed by the hematoma is unlikely to be salvaged because the immediate dissection of blood through this area causes direct and rapid tissue destruction[14]. However, the degenerative cascades and secondary events in regions adjacent or distal to the hematoma are feasible targets, as recent studies show that cell death occurs in these regions over hours to weeks[5, 62, 75]. Thus, this tissue is potentially salvageable by countering degenerative events, which include neurotoxicity induced by the coagulation cascade (e.g., thrombin production) and degenerating red blood cells (RBC), oxidative damage, disruption of the BBB, edema formation, and inflammation[46]. Notably, ICH and ischemia share many mechanisms of injury and thus it is not surprising that many treatments found to be effective in ischemia are being tested for treating ICH. However, there are also fundamental differences between ischemia and hemorrhage. For instance, intracerebral blood has direct and indirect toxic effects, such as through the production of thrombin, which stimulates edema, inflammation, and the generation of free radicals[43]. Another difference is that the evidence for an ischemic penumbra surrounding the hematoma core is controversial[76, 77], whereas it is well established to follow ischemia. Perhaps this is because reductions in CBF in peri-hematoma tissue may indicate reduced metabolic demand, and not necessarily ischemia. Thus, although it makes sense to test treatments that are efficacious in ischemia, alternative approaches to ICH must also be investigated.

1-5a Surgery

In the past several years, a number of treatments options have gained considerable attention. Surgical removal of the hematoma may limit the extent of cell death and secondary degenerative processes after ICH by reducing space-occupying effects of the hematoma and subsequent elevations in intracranial pressure, by improving CBF, and by removing potentially toxic blood breakdown products. Although some benefit was obtained in rats[78], findings in pigs are contradictory[79, 80]. Importantly, the recently failed Surgical Trial in ICH suggests that surgery does not benefit ICH patients[29]. However, surgical removal of the hematoma may be more effective for some types of hemorrhagic stroke, such as a lobar or cerebellar ICH.

1-5b Anti-Hypertensive Treatment

Acute hypertension may predispose ICH patients to hematoma expansion, and therefore, blood pressure (BP) reduction could reduce hematoma expansion and subsequent death and disability. However, some controversy still exists over BP management in ICH patients[81]. Lowering BP may worsen neurological outcome by inducing adverse events such as ischemia in the tissue surrounding the hematoma. Recent studies have assessed whether reducing elevated BP in ICH is safe and feasible. Qureshi and colleagues[82] recently found that reducing and maintaining blood pressure in accordance with the guidelines of the American Heart Association (e.g., maintaining mean arterial pressure < 130 mmHg while keeping cerebral perfusion pressure >70 mmHg) is feasible and well tolerated in ICH patients. A multi-center randomized trial to assess the efficacy of anti-hypertensive treatment initiated within 6 h of ICH is currently underway[82].

1-5c Hemostatic Therapy

Early hemostatic therapy using human recombinant factor VIIa (rFVIIa; NovoSeven®) is a promising treatment for ICH patients who undergo hematoma expansion[27, 28]. A recent study reported reduced mortality, smaller hematoma size and improved neurological outcome in patients who received rFVIIa within three hours of ICH[28]. Although Phase III clinical trials have not yet been completed, if rFVIIa is found to be safe and effective for ICH, it may become the standard care for ICH, and the equivalent to tPA in the management of ischemic stroke[83]. However, rFVIIa will not likely be used for all ICH patients because (as is the case for tPA) only a small percentage of patients will receive medical treatment within 3 h of ICH. Furthermore, the increased risk of thromboembolic complications raises concerns about the safety of rFVIIa, especially for the 70% of ICH patients who do not experience hematoma growth.

1-6 Recommendations for Experimental Stroke Studies

Many therapeutic compounds (e.g., MK-801, NBQX) found to be effective in experimental stroke studies were advanced to clinical trials with anticipation that they would be effective in stroke patients. Unfortunately, all of these clinical trials failed, leading to questions about the value of animal stroke models, the design of clinical stroke trials, and whether cytoprotection is a promising treatment strategy. The failure of translation from the bench to the bedside is due to limitations in the design of both experimental and clinical studies. A number of recommendations have been made to improve the quality of experimental ischemia cytoprotection (cell saving) studies[84, 85], and many of these are relevant to evaluating cytoprotection in ICH. For instance, the optimal dose and duration of treatment must be identified. Furthermore, longer delays to

initiation of drug administration (up to several hours after stroke) should be examined. Whereas most cytoprotection studies use young-adult, male, healthy animals, efficacy should also be assessed in aged animals of both sexes, and with co-morbid conditions such as hypertension and diabetes. Studies in neonates mimicking periventricular / intraventricular hemorrhage are also needed[86]. Most studies rely only on histological measures to assess outcome, yet few examine both white matter and gray matter injury. Markers of injury such as the size of the hematoma or amount of edema are often used to gauge treatment efficacy. However, reductions in edema, for instance, may neither improve long-term functional outcome nor permanently reduce injury. Thus, investigators much determine how biochemical markers of injury relate to cell death, and whether targeting these processes improves long-term outcome.

In addition to histological outcome, functional assessment is a priority as it is the clinical endpoint of greatest concern. Thus, a battery of functional tests appropriate for each model should be used. As cytoprotectants may provide only transient benefit, late assessment of histological and functional outcome should be performed. Finally, rigorous pre-clinical testing requires the evaluation of cytoprotectants using multiple clinically relevant models and several species, for instance in both rodent and porcine, and eventually primate models. The identification of truly effective cytoprotectants for ICH warrants a similar approach.

1-7 Animal Models of ICH

The majority of cytoprotection studies use rodent models of ICH, but other species, such as pig[40], are also used. The common methods of inducing ICH in rodents are the bacterial collagenase[87] and autologous blood injection methods[88].

Intraparenchymal injection of autologous blood is most commonly used to model ICH in rodents[37]. Although this model is routinely used in pathophysiologic, biochemical, and behavioural studies, it does not have a ruptured blood vessel as the underlying cause, and does not reproduce the active bleeding event that occurs in human ICH. There are several other limitations with this model. First, except for brain edema in the acute phase, it is difficult to quantify the extent of brain injury after ICH because only a very small cavity exists after the clot is absorbed[89]. Second, the mild insult produced causes behavioural deficits that mostly resolve by 3-4 weeks following ICH[89, 90]. Thus, it is difficult to assess long term (e.g., 30 days) histological and behavioural function in this model, as well as small cytoprotective effects.

Bacterial collagenase digests the Type IV collagen with the basal lamina of blood vessels, leading to hemorrhaging[87]. The progression of cell death[62] and behavioural deficits[91] are well characterized in this model. In accordance with others' work[62, 87], we have determined that bleeding continues for 4-6 h after the onset of ICH in this model[92]. Furthermore, it is possible to affect the amount of bleeding, and thereby significantly impact long-term outcome. For example, 17-ß estradiol pretreatment reduces intraparenchymal bleeding and brain injury after ICH[93], while early hypothermia treatment aggravates it[94]. Thus, this model may be useful for studying the impact of rebleeding or continued hemorrhage, which frequently (> 30%) occurs in ICH patients[95]. However, the widespread degradation of the endothelial basement membrane of blood vessels differs from that of a spontaneous ICH in humans, and the enzyme may be neurotoxic to parenchyma.

Other models of ICH, such as implanting an inflatable balloon[88], or injecting components of blood (e.g., thrombin) into the parenchyma[96] are used principally to characterize pathological processes of ICH, and less often for evaluating cytoprotectants. Due to the limitations of these animal models, the development of other relevant models of ICH is clearly needed for experimental ICH studies.

1-8 Experimental Approaches for ICH

A variety of cytoprotectants have been tested experimentally in rodents (for review, see [97]). Most therapies aim to reduce secondary consequences of ICH because the primary injury occurs rapidly (over the first few hours) and is not likely salvageable. Such therapies include anti-inflammatory agents[98-101], free-radical scavengers[102, 103], thrombin inhibitors[104], anti-apoptotic agents[105], and others. Edema occurring in the acute phase of ICH has been reduced by a number of treatments (e.g., celecoxib, deferoxamine[63, 104, 106]), however, no drug has persistently reduced the amount of tissue lost. Furthermore, functional improvements have been limited to tests of gross neurological function (e.g., neurological deficit score; NDS), and have not been found in other tests (e.g., skilled reaching) over the long term. These failures are likely due to the fact that most putative cytoprotectants target only one mechanism of injury. However, due to the number of deleterious processes that contribute to hemorrhagic injury, effective cytoprotection may only be achieved with treatments that affect multiple mechanisms of injury (excitotoxicity, free-radical mediated damage, inflammation, etc.). One such treatment is mild and prolonged hypothermia.

1-9 Hypothermia

1-9a Early Literature

Hypothermia is defined as a body temperature below 36 °C and is classified as mild (32 – 36 °C), moderate (28 – 32 °C), or severe (< 28 °C). Medical use of hypothermia has been documented since the 1940's for diseases such as schizophrenia,[107] cancer,[108] vascular surgery,[109] and cardiac arrest[110, 111]. Experimentally, hypothermia has been studied in monkeys, dogs and cats *during* traumatic brain injury and ischemic stroke (for review, see[112]), with reports of increased survival, improved neurological outcome, and reduced brain injury (for example, see[113, 114]). Early experimental studies have also assessed the effects of *delayed* hypothermia (i.e., induced after the insult), which is a more clinically relevant intervention for experimental brain injury, and is the topic of this thesis. Researchers recognized that hypothermia initiated *after* an insult would likely require a longer duration of treatment to be effective. Thus, these studies tested prolonged periods of hypothermia, but often reported negative results. For example, Michenfelder and colleagues found that prolonged (e.g., 48 h) hypothermia (29 °C) following middle cerebral artery occlusion (MCAO) in monkeys[115] and cats[116] were lethal. Notably, hypothermia resulted in close to 100 % mortality, compared to less than 33 % mortality in untreated animals or historical controls. In general, these and other studies used moderate (28 – 30 °C) and prolonged (18 – 72 h) hypothermia and were plagued by high mortality, which generally occurred during or soon after rewarming[115, 117, 118]. This early work revealed several deleterious side effects of hypothermia including: body temperature drift, cardiac irritability and ventricular fibrillation, the appearance of shock and death during rewarming, and kinetic inhibition of clotting factors leading to coagulopathy[119]. Medical use of hypothermia declined drastically after these reports

because of these potentially devastating side effects and several other reasons. For instance, early experimental and clinical studies were not convincing as they were often plagued by design flaw including small sample size and lack of appropriate controls[112]. Negative findings may also relate to rapid rates of rewarming causing rebound ICP and death, or to species differences. Perhaps hypothermia is better tolerated in humans and rodents than in other species (e.g., dogs) used in those studies.

1-9b Hypothermia Revisited

A resurgence of interest in hypothermia occurred in the 1980's, with the use of *mild* hypothermia during global and focal ischemia in rodents (for review, see[112]). Mild hypothermia was used in order to avoid the deleterious side effects that occur with greater reductions in temperature. These studies provided overwhelming evidence that intraischemic hypothermia provides near-permanent protection for ischemia. For instance, intraischemic hypothermia provided lasting (weeks to months) histologic and functional protection in global ischemia (e.g., [120, 121]), and intraischemic hypothermia is considered to be the gold standard of neuroprotection in stroke studies. Furthermore, its efficacy is unmatched by any pharmacological neuroprotective agent. However, there is a need for postischemic interventions, as most stroke patients do not receive treatment until several hours after their stroke. Initially, there was some controversy over the value of postischemic hypothermia, likely due to the fact that early studies tended to use short (e.g., a few hours) durations of hypothermia and reported little or transient protection[122, 123].

1-9c Prolonged and Mild Postischemic Hypothermia for Global Ischemia

It was evident that prolonged mild postischemic cooling is needed to obtain lasting protection, thus leading to the study of prolonged postischemic hypothermia interventions for stroke. In several global ischemia studies[124, 125], Colbourne and colleagues examined factors such as the duration of cooling and delay to treatment, which were thought to affect the efficacy of hypothermia. Because prolonged use of anesthetic led to excessive mortality, hypothermia was produced manually in these studies, using fine water misters and fans to cool, and infrared lamps to warm. First, gerbils were subjected to 3 or 5 minutes of forebrain ischemia and were then maintained at normothermia or subjected to prolonged hypothermia (12 h at 32 °C) starting 1 h after ischemia[124]. Hypothermia provided moderate protection against the 5 minute insult at a 7 day survival, but protection declined with a long (e.g., 30 day) survival. However, hypothermia provided near-total preservation of CA1 cells and reduced behavioural (e.g., open-field habituation) impairments following the 3 minute insult. Clearly, the duration of ischemia affected the degree of hypothermic protection. Subsequently, the efficacy of 24 h of hypothermia was tested following 5 minutes of forebrain ischemia. Near-perfect protection of CA1 was detected at a 1-month survival, indicating that prolonged hypothermia is more effective than shorter (e.g., 12 h) durations, especially against more severe insults. The same hypothermia treatment (24 h at 32 °C; 1 h delay) provided substantial protection at a 6 month survival, although protection was less than at 1-month. These findings confirm that delayed and prolonged mild hypothermia provides significant and long-lasting protection for ischemia. The influence of delay to treatment (1 vs. 4 h) and degree of hypothermia (32 vs. 34 °C) were also assessed[125]. Cooling to 32 °C was

more efficacious than 34 °C, and although there was significant protection with the 4 h delayed intervention, it was not as effective as hypothermia initiated at 1 h.

These systematic studies in global ischemia provided the foundation for hypothermia research in subsequent ischemia studies, and provided considerable evidence to suggest that hypothermia would effectively treat ischemic injury in humans. In fact, two recent clinical studies reported that a hypothermia treatment similar to that tested in rodents (e.g., 32 °C for > 24 h) reduces mortality and improves functional outcome after out-of-hospital cardiac arrest[126, 127].

1-9d Prolonged and Mild Postischemic Hypothermia for Focal Ischemia

The efficacy of hypothermia against focal ischemic injury is of great clinical interest. Many studies have examined the effects of brief (e.g., 1 to 4 h) periods of hypothermia[128-131] and found that longer bouts are most effective[130]. However, as in global ischemia, brief cooling provides little to no benefit at protracted survival times[130]. Only a handful of studies have assessed the efficacy of prolonged postischemic hypothermia in focal ischemia[132-135]. Colbourne and colleagues used an automated system of inducing hypothermia in awake and freely moving rodents[136]. For example, using the intraluminal suture occlusion model of MCAO in the rat, 48 h of hypothermia (brain temperature of 34 °C) induced 30 minutes after the onset of reperfusion significantly reduced cortical and striatal brain injury at two months[133]. Similarly, 48 h of hypothermia (core temperature of 33 °C for 24 h, then 35 °C for 24 hours) prevented skilled reaching deficits and reduced the volume of tissue lost at one month[132] after MCAO produced via the clip occlusion model. In humans, hypothermia reduces edema in massive ischemic strokes[137-139], and is beneficial as an adjunctive therapy to rt-PA[140-142].

In summary, these studies provide overwhelming evidence that hypothermia provides remarkable and long-lasting neuroprotection when induced after global and focal ischemia. Although the optimal hypothermia treatment has not yet been defined, the efficacy of hypothermia depends on several factors. For instance, a prolonged period of hypothermia affords greatest protection, whereas a shorter bout may provide little or transient benefit. Furthermore, hypothermia is most efficacious when induced as early as possible after ischemia. Although some benefit occurs when hypothermia is delayed for up to 12 h after ischemia, the amount of protection decreases as the delay to treatment increases. The degree of mild hypothermia also warrants consideration, as greater reductions in body temperature (within a safe range) are more efficacious.

1-10 Mechanisms of Hypothermia Protection

Hypothermia protects against the effects of cerebral ischemia by multiple mechanisms (for review, see[112, 143, 144]). First, hypothermia decreases cerebral metabolism[145] and energy demand soon (minutes to hours) after ischemia and may therefore preserve ATP stores and reduce intracellular acidosis[146, 147]. Immediately following ischemia, excessive release of glutamate and other excitatory amino acids increases intracellular calcium concentrations through activation of NMDA and AMPA receptors, leading to ischemic cell death via cytotoxicity[148-150]. A decrease in intracellular calcium concentration (by hypothermia) may suppress the generation of oxygen free radicals, thereby protecting cell membranes and organelles from ischemic and reperfusion injury[151, 152]. Inhibition of protein synthesis also causes ischemic cell death[153]. Hypothermia promotes the recovery of normal protein synthesis and degradation following ischemia, which helps to maintain neuronal viability[154, 155]. Blood-brain barrier

disruption following ischemia[156] may be attenuated by hypothermia, which would lessen vasogenic edema and intracranial pressure[157, 158], and may also reduce the rate of hemorrhagic transformation following ischemia[159]. Furthermore, maintenance of the BBB prevents toxic substances in the blood from entering the brain[156]. Hypothermia also inhibits the inflammatory response after ischemia, and suppress the infiltration of inflammatory molecules (e.g., cytokines, neutrophils)[160]. Finally, hypothermia may directly interfere with genes involved in apoptotic cell death following ischemia, or may somehow interrupt apoptotic cell death pathways, to reduce neuronal injury[161].

1-11 Hyperthermia after Ischemia

Increased body temperature markedly exacerbates injury in acute stroke. Elevated body temperature on admission[162] or in the days following ischemia[163] is significantly associated with increased morbidity and mortality. Studies of global[156, 164] and focal[165, 166] ischemia in rodents have confirmed that even small increases in body temperature critically affect outcome. Hyperthermia acts through several mechanisms to aggravate injury, including increased release of neurotransmitters[167], BBB breakdown[156, 168], increased free radical production[152], ischemic depolarizations in the penumbra[169], cytoskeletal proteolysis[170], and impaired energy metabolism[171] (for review, see[172]). In light of the overwhelming evidence that hypothermia improves stroke outcome and hyperthermia exacerbates injury, it is recommended that body temperature should be closely monitored and maintained at normothermic temperatures for several days following stroke[162, 172, 173].

1-12 Hypothermia for Intracerebral Hemorrhage

Given that hypothermia provides significant and persistent cytoprotection in models of global and focal ischemia, it is reasonable to expect that hypothermia would effectively treat ICH as well. Indeed, ICH and ischemia share many mechanisms of injury, including edema and elevations in intracranial pressure, inflammation, oxidative damage, BBB disruption, excitotoxicity, and possibly ischemia in the tissue surrounding the hematoma. However, the efficacy of mild and prolonged hypothermia has not been tested after experimental ICH. In fact, there is very little research on the effects of temperature manipulation after ICH. In rats, prolonged (24 h) hypothermia reduces edema formation following striatal thrombin injection[174, 175]. Edema and BBB disruption are also reduced by delayed hypothermia after collagenase-induced ICH[176]. Intra-operative hypothermia has been applied safely in some subarachnoid hemorrhage[177] and ICH patients[178], but the efficacy of hypothermia has never been assessed in clinical trials.

There were three main objectives of this thesis. First, I evaluated the efficacy and mechanisms of mild and prolonged hypothermia after a striatal ICH in rats. Second, I assessed whether mild to moderate hyperthermia would affect ICH outcome. Third, I examined spontaneous recovery in two models of striatal ICH in order to identify an appropriate battery of behavioural tests sensitive to long - term functional deficits in each model. As described earlier, a number of recommendations have been made to improve the quality of preclinical stroke studies, which include using clinically relevant (e.g., delayed) interventions, multiple animal models, rigorous assessment of functional outcome as well as histology, and protracted survival times. In this thesis, I created striatal ICH injury as this most commonly occurs in ICH patients. I evaluated the

functional (e.g., behavioural) and histological effects of delayed (1-12 h) hypothermia and hyperthermia treatments. Prolonged and mild hypothermia was induced using an automated system for systemically cooling rodents[136]. In each study, functional outcome was assessed over one month following ICH using several behavioural tests (e.g., skilled reaching, walking, neurological deficits) known to be sensitive to ICH injury. Histological outcome (e.g., volume of injury) and mechanisms of protection (e.g., reduced inflammation and edema) were also assessed. Finally, the last two studies examined spontaneous recovery in the bacterial collagenase and whole blood models of ICH, and identified behavioural tests sensitive to long-term deficits in each model. Overall, this thesis comprehensively evaluated the therapeutic potential of mild and prolonged hypothermia for ICH in rats. The knowledge gained from these studies may be used to further improve preclinical ICH studies and facilitate the development of more effective hypothermia treatments for ICH.

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Chapter 2: Early and prolonged hypothermia does not favorably affect intracerebral hemorrhage in rats

A version of this chapter has been published. MacLellan, C.L., Shuaib, A., and Colbourne, F. 2002 *Brain Research*, 958: 192-200.

2-1 Introduction

Intracerebral hemorrhage (ICH), which commonly occurs in the striatum, thalamus, cerebellum and pons, accounts for approximately 10 - 15% of strokes in Western populations[1] and is one of the most devastating types of stroke. Indeed, the 30-day mortality rate is ~50%, and neurological recovery in survivors is often poor[2]. Neurological deficits result from direct tissue destruction, space-occupying effects of the hematoma, a reduction of cerebral blood flow in surrounding tissue[3], and cerebral edema[4]. A variety of treatment strategies have been tested such as the surgical removal of the hematoma[5-7], and treatment of raised intracranial pressure (ICP[8]). Experimental treatments aim to reduce either tissue damage or the secondary consequences of an ICH, such as inflammation[9, 10] or raised ICP. Unfortunately, ICH remains a clinically difficult problem to treat[11] and experimental studies have so far failed to show exceptional benefit from any therapy.

Delayed hypothermia reduces ischemic injury in rodents and promotes functional recovery[12, 13]. For example, mild postischemic hypothermia, when prolonged (e.g., 24 hr), provides effective and long-lasting protection of hippocampal CA1 neurons in both gerbil[14, 15] and rat[16] models of global ischemia. Delayed hypothermia also reduces infarct size and functional impairments following middle cerebral artery occlusion (MCAO) in rats[17-19]. Again, protection appears to be better with protracted durations of mild hypothermia (e.g., 2 days[17, 18]). Prolonged hypothermia is a feasible treatment for stroke[20, 21] and global ischemia[22] in humans and is currently under investigation. Hypothermia markedly decreases ICP in patients with traumatic brain injury (TBI[23, 24]) and stroke[21, 25]. In rats, Kawai and colleagues[26] showed that prolonged (24 hr)

hypothermia (30°C initially, then rising to 35°C within 10 hrs) reduced vasogenic edema formation following injection of thrombin into the basal ganglia, possibly by lessening the inflammatory response and BBB breakdown. Accordingly, we hypothesized that prolonged mild hypothermia, as previously found to be especially effective for treating global and focal ischemia and reducing edema formation, would reduce the functional deficits and histological damage of a relatively mild hemorrhagic stroke in rats.

In a model of ICH developed by Rosenberg and colleagues[27], the infusion of bacterial collagenase into the rat brain disrupts the basal lamina of cerebral vasculature and causes bleeding into surrounding brain tissue. The progression of ICH in this model has been well characterized using magnetic resonance imaging[28], and outcome has been assessed with behavioral testing (e.g., skilled reaching) and histopathological analyses (e.g., lesion size)[9, 29, 30]. Thus, we used this model of striatal hemorrhagic stroke to test whether delayed and prolonged mild hypothermia would reduce the size of the hemorrhagic lesion and the neurobehavioral consequences.

2-2 Materials and Methods

All procedures used in this study were in accordance with the Canadian Council on Animal Care guidelines, and were approved by the Biosciences Animal Policy and Welfare Committee at the University of Alberta.

2-2a Animals

In total, thirty-six male Wistar rats (Charles River, Montreal, Quebec, Canada) were entered into this study. Rats weighed between 250 and 350 g (~ 3 – 4 months old) at the start of the experiment, which was the Montoya staircase training.

2-2b Montoya Staircase Training

Rats were food deprived to 90% of their free-feeding weight over the course of three days and weighed daily to ensure stability at 90%. They were trained in the staircase test, which measures independent forelimb reaching ability[31], over 15 days (two 15-minute trials per day separated by 4 – 5 hr, five days per week[17]). Rats were considered to have acquired the task when they retrieved at least nine pellets (45 mg each, Bio-Serv, Frenchtown, NJ, U.S.A) per side out of a possible 21 pellets on three consecutive days during training. Regardless all rats were trained for 30 trials. Upon completion of training rats were returned to ad lib feeding. Two rats were excluded because of failure to reach this level of performance; another was excluded for being very difficult to handle.

2-2c Core Temperature Telemetry Probe Implantation

Six days after the end of staircase training, rats were briefly anesthetized with isoflurane anesthesia (4% induction; 1.5 to 2% maintenance in 70% N_2O, 30% O_2) and telemetry core temperature probes (model TA10TA-F40; Data Sciences, St. Paul, MN, U.S.A.) were implanted into the peritoneal cavity. The abdominal wound was treated with a local anesthetic (Marcaine; Sanofi Canada, Inc., Markham ONT, Canada) and sutured closed. Rats were then housed individually upon receivers (RPC-1; Data Sciences) interfaced to a computer running DataSciences telemetry software which sampled temperature every 30 seconds. Data from the complete day prior to ICH or sham surgery served as a baseline. Temperature data were analyzed with ANOVA followed by Scheffé's correction for individual comparisons (SPSS, v 11.0).

Core temperature, when measured with telemetry probes, correlates well with brain temperature in normal awake or anesthetized rats. For instance, we[32] compared core

and brain temperatures in awake rats, both measured with telemetry probes, and found r = 0.97 ($P < 0.0001$). Similar correlations are found in anesthetized rats as long as cerebral blood flow is not impaired. Notably, however, brain temperature is ~0.7°C lower than core temperature. Brain temperature was not measured in this study because of the desire to maintain a closed cranium, and therefore to allow ICP to rise after the hemorrhagic stroke, and due to technical problems with securing a probe on the head while allowing access to the striatum for collagenase infusion (see next section).

2-2d Intracerebral Hemorrhage / SHAM Surgery

Rats were anesthetized with isoflurane (1.5 - 2% maintenance) and placed in a stereotaxic frame. During anesthesia (~30 minutes) core temperature was maintained near 37°C (normothermia) with a heating pad. Following a midline scalp incision (earlier treated with Betadine), a small burr hole was made 3 mm contralateral to preferred paw (as determined by performance in the staircase test; random if no clear preference) and 0.2 mm posterior to bregma. A 26-gauge needle (Hamilton syringe, Hamilton Company, Reno, Nevada) was then lowered 6 mm below the surface of skull and we infused 0.7 µl of sterile saline containing 0.14 U bacterial collagenase (Type IV-S, Sigma Chemical Co., Oakville, ONT, Canada) to produce intracerebral hemorrhage (ICH) or sterile saline alone (SHAM procedure) into the striatum over a period of 5 minutes. The needle remained in place for an additional 5 minutes following infusion. A metal screw (model MX-080-2, Small Parts Inc, Miami Lakes, FL, USA) was inserted to the thickness of the skull bone thus sealing the burr hole and allowing for subsequent ICP elevations due to the hemorrhage. The scalp wound was then infiltrated with Marcaine and closed with staples. All rats were shaved (part of abdomen and back) to facilitate subsequent

hypothermia treatment or to prevent knowledge of group identity in the normothermic groups. Importantly, prior to surgery all rats were randomly assigned to SHAM or ICH groups and then randomly assigned to either normothermic or hypothermic conditions.

The dose of collagenase was chosen to produce a mild – moderate sized lesion and was based upon studies by Peeling and colleagues[33]. A mild lesion was presumed to be more amenable to treatment with hypothermia than a severe insult, thus maximizing our chances of detecting beneficial effects.

2-2e Post-Surgery Temperature Control

The ICH (N = 8 included) and SHAM (N = 5) animals were allowed to freely regulate their own temperature. ICH+HYPO (N = 8) and SHAM+HYPO (N = 6) groups were slowly cooled, starting at 1 hr after ICH induction, by a rate of 1°C per 30 minutes to a core temperature of 33°C and maintained at this level for 24 hours. Rats were then slowly warmed (1°C / 30 min.) to 35°C and kept at that temperature for an additional 24 hours. Following this, core temperature was maintained between 36°C and 37°C (low normothermia range) for 24 hours. Temperature was precisely (± 0.3°C) regulated using a servo-controlled system that employed fans and fine water misters for cooling and infrared lamps for warming[34]. Temperature was monitored in all animals for 7 days after surgery before telemetry probes were quickly removed under isoflurane anesthesia.

Three additional rats were excluded due to technical problems (e.g., significant bleed upon insertion of needle). Another three rats were later excluded due to aberrant lesion location. No mortality occurred during this experiment.

2-2f Behavioural Testing

Limb-use asymmetry test

Rats were put in a transparent cylinder (45 cm in height and 33 cm in diameter) for five minutes and allowed to spontaneously explore the cylinder on days 7, 14, 21, and 28 following ICH / SHAM surgery. A video camera set up below the cylinder recorded all forelimb placements, which were recorded and analyzed according to Tillerson et al.[35]. Briefly, forelimb use was noted for initiating a rearing movement, exploration of cylinder walls, and landing after a rearing movement. A push-off was considered the independent use of either forelimb (or simultaneous use) when a rearing movement began. Wall exploration included the initial contact of a forelimb with the wall and subsequent single-limb contacts during lateral movements across the wall while maintaining a vertical posture. The use of the opposite limb while maintaining an initial wall contact was scored as a simultaneous use. Landings included the use of either forelimb (or both) to land after a rearing movement. Push-off, wall exploration, and landing forelimb placements were scored independently and expressed in terms of: (1) the percentage of impaired limb use in relation to the total number of limb-use movements, (2) the percentage of non-impaired limb use in relation to the total number of limb-use movements, and (3) the percentage of simultaneous use of forelimbs or "co-use" relative to the total number of limb placements. This data were analyzed with ANOVA with Scheffé's correction for individual comparisons.

Horizontal Ladder-Walking Test

On days 7, 14, 21, and 28 post-surgery, rats were tested on the horizontal ladder-walking test[36]. Each session consisted of three consecutive trials in which the rat walked across a horizontal ladder with randomly spaced "rungs" or bars (spacing between bars ranged from 1 – 3 cm). For each limb, the total number of steps and the slips (limb falls

through bars) made to traverse a 0.5 m segment was analyzed with ANOVA with Scheffé's correction for individual comparisons.

Staircase Test

Rats were food-deprived to 90% of their free-feeding weight prior to staircase testing which consisted of 10 trials (two trials per day) on days 21 to 25 after ICH / SHAM surgery. The total number of pellets retrieved on each side, which was expressed as a percent of baseline (average of the last 10 training trials), was recorded for each trial and analyzed with ANOVA with Scheffé's correction for individual comparisons.

2-2g Histology

Rats were allowed to survive for 30 days following ICH or sham operation. They were euthanized with an overdose of Somnotol (80 mg/kg) and were transcardially perfused with 0.9% saline and then 10% formalin. Forty μm coronal sections were taken with a cryostat. Sections were taken every 200 μm starting at +1.7 mm to bregma and extending back to –4.8 mm to bregma. Sections were stained with Cresyl Violet and using Scion Image J 4.0, the volume of the lesion plus atrophy (e.g., ventricular enlargement) was quantified and expressed as follows:

Volume of tissue lost = remaining volume of normal hemisphere – remaining volume of injured hemisphere.

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.

Volume of tissue lost (i.e., a combination of the hemorrhagic infarct, cavity and ventricular enlargement) was analyzed with a one-way ANOVA.

In addition to calculating the volume of tissue lost, we estimated tissue destruction by the methods of Altumbabic et al.[29]. Briefly, the area of the contralateral and ipsilateral striatum as well as the hematoma was measured. Striatal loss was considered to be the difference between the contralateral striatum and the remaining ipsilateral striatum (ipsilateral striatum – hematoma).

2-3 Results

2-3a Temperature

Baseline core temperature, measured with the telemetry probes, was collected the day before ICH / SHAM surgery and was similar among groups (37.0°C ± 0.02 SEM) and similar to previous studies. Temperature during and after surgery (Figure 1) was regulated as desired (see Materials and Methods). For 12 hours after surgery, ICH rats were slightly warmer than SHAM rats (37.5°C vs. 37.2°C respectively; ANOVA on mean temperature: $P = 0.003$).

2-3b Body Weight

The core probe implantation procedure caused a small drop (~4 g) in body weight, which was similar in all groups (data not shown). The ICH and SHAM groups had similar postoperative (days 4, 7, 14, 21 and 28) weights (% surgery day) after the infusion procedure. However, hypothermia treatment in the SHAM+HYPO (vs. SHAM; $P \leq 0.045$) and ICH+HYPO (vs. ICH; $P < 0.001$) groups did result in statistically significant weight loss on days 4 and 7, but this never exceeded 10% of their pre-surgery body

weight. All groups had regained any lost weight and were not significantly different on days 14, 21 and 28 ($P \geq 0.345$). Induced weight loss (~9 % of baseline overall) during staircase testing was not significantly different among groups.

2-3c Staircase Testing

All groups performed similarly on the training phase of the staircase test (data not shown). As expected, there was no significant difference ($P \geq 0.726$) in the food pellet retrieval (% baseline which was defined as the average performance on the last 10 trials of training) of the SHAM and SHAM+HYPO groups with either the ipsilateral or contralateral forelimb (Figure 2). Thus, these groups were combined for subsequent analysis. Neither the ICH nor the ICH+HYPO groups had statistically significant ipsilateral reaching impairments ($P \geq 0.083$), whereas both obtained significantly less pellets than SHAMs (combined groups) with the contralateral forelimb ($P \leq 0.010$). Hypothermia did not lessen this deficit ($P = 0.967$).

2-3d Horizontal Ladder-Walking Test

The total number of steps taken to cross the 0.5 m length of horizontal ladder was very similar among groups (data not shown). There were no significant group effects for any limb ($P \geq 0.091$) although there was a trend towards an impairment (higher % of falls) with the contralateral forelimb in the ICH and ICH+HYPO groups (vs. SHAMs; Figure 3). These group comparisons did not reach statistical significance with the Scheffé's test ($P \geq 0.153$) or even with simple contrasts ($P \geq 0.056$), which did not correct for multiple comparisons (i.e., less likely to make a Type II error).

2-3e Limb-use Asymmetry Test

There were no significant differences between SHAM and SHAM+HYPO groups

on measures of paw placement on the wall, on push off and on landing (ANOVAs; $P \geq 0.385$); thus these groups were combined for subsequent analyses. While there were some trends towards diminished usage (vs. SHAMs) of the contralateral forelimb in ICH groups (e.g., placement on wall; Figure 4), there were no significant group main effects for usage of the contralateral paw ($P \geq 0.133$), ipsilateral paw ($P \geq 0.127$) or co-usage ($P \geq 0.555$) on paw placement on the wall, and on landing. For paw placement on push off we found no significant group effect for the contralateral limb ($P = 0.112$) or co-usage ($P = 0.071$), but did find a significant group effect for the *ipsi*lateral limb ($P = 0.048$), which was due to the ICH+HYPO group using their ipsilateral limb more frequently than SHAMs on weeks 1 ($P = 0.042$) and 4 ($P = 0.009$) only. There were no significant differences between these groups on weeks 2 and 3; nor were any differences found between the ICH and ICH+HYPO groups. Accordingly, this test was not very useful in distinguishing among groups.

2-3f Volume of Lesion

Injection of collagenase in ICH rats resulted in a mean total tissue loss of 23.1 $mm^3 \pm 4.5$ SEM. This largely included striatum, more medially (Figure 5) but often lateral as well. Sometimes the lesion affected thalamus, globus pallidus and the internal capsule. Notably, prominent ventricular dilation was seen ipsilateral to the lesion and somewhat anterior and posterior to the obvious hemorrhagic stroke focus. Post-ICH hypothermia did not significantly affect the volume of tissue lost (22.2 $mm^3 \pm 3.3$ SEM; $P = 0.982$ vs. ICH). Sham groups did not sustain any damage aside from the needle tract except for one SHAM rat (noted in Methods), which had a significant cortical lesion. Since this animal was noted to have a large bleed upon needle insertion it was excluded.

Using the methods of Altumbabic and colleagues[29], tissue loss (in mm^2) was estimated using the section with the maximum hematoma diameter. There was a strong correlation ($r = 0.84$; $P < 0.001$) between the area of striatal loss calculated from that single section and the total volume of injury calculated from sections encompassing the entire lesion (36 sections per rat from ICH and ICH+HYPO groups). In order to determine whether it is necessary to take sections every 200 μm, the volume of lesion was also estimated using sections 400 μm and 600 μm apart. Lesion volume (all ICH and ICH+HYPO rats) was similar when sections were taken at 200 μm (22.7 mm^3), 400 μm (21.7 mm^3) and 600 μm (21.9 mm^3) intervals ($F_{2, 45} < 1$).

2-4 Discussion

Prolonged mild hypothermia failed to reduce either the volume of tissue lost following ICH or the behavioral deficits (skilled reaching) that accompany unilateral striatal injury. This is in stark contrast to the beneficial effects of similar hypothermia treatments induced hours after global[15, 16] and focal cerebral ischemia[17, 18] and the use of hypothermia after thrombin injection to reduce edema[26]. The infusion of bacterial collagenase resulted in a mild to moderate-sized ICH affecting largely the striatum, but extending to include thalamus, globus pallidus and internal capsule. Most tissue destruction, previously characterized by Del Bigio and colleagues[28], occurs over several hours. Thus, our hypothermia treatment, which was initiated 1 hr after infusion of collagenase, should be considered an early intervention. We intentionally used a mild to moderate insult and early induction of hypothermia to maximize the likelihood of finding a beneficial effect while maintaining clinical relevance (i.e., delayed induction of therapy). Contrary to our expectations, hypothermia had no discernable effects on the

volume of tissue lost or the skilled reaching deficit. There are several likely explanations: 1) this type of hemorrhagic insult does not have a substantial ischemic component and thus hypothermia, an anti-ischemic therapy, would be ineffective, 2) most injury occurs too rapidly to be effectively treated by delayed treatments or the insult was too severe, 3) the therapy was sub-optimal (e.g., too brief) and 4) benefit was only transient.

The severity of an insult dramatically affects the amount of neuroprotection observed with any treatment. For example, a 12-hr duration of hypothermia barely reduces CA1 cell death after 5 minutes of normothermic forebrain ischemia in gerbils, whereas near-total protection is observed after a 3-minute insult[14]. Thus, one could argue that a milder hemorrhagic insult than that presently used might be amenable to hypothermia therapy. However, the ICH insult used was relatively mild since it did not result in significant (or at least persistent) impairments on two of our behavioral tests (i.e., the horizontal ladder and the cylinder task) or in any mortality. Furthermore, the impairment observed in the staircase test (Figure 2) was similar to that observed after cortical infarction after distal MCAO, an insult that *is* amenable to delayed hypothermia treatment[17]. Accordingly, our insult was not likely too severe. Indeed, it is possible that our insult was *not* sufficiently severe to properly assess hypothermia. For example, prolonged cooling might attenuate those deleterious events (e.g., blood-brain barrier breakdown, edema, raised ICP) that accompany severe ICH as it has been shown to do after thrombin injection[26]. While such changes may occur following the present ICH insult, they were not sufficiently severe to result in mortality and may not have contributed substantially to outcome. Consequently, it is possible that hypothermia therapy may not provide benefit after mild hemorrhagic insults, but may be indicated for

severe insults (e.g., those with delayed edema and ICP elevations), which we plan to assess.

The duration of hypothermia markedly affects neuroprotection. For example, 12 hr of hypothermia provides little persistent protection in CA1 after global ischemia while a 24-hr duration provides near-total protection[14]. As we used over two days of mild hypothermia, which in models of global and focal ischemia provide substantial functional and histological protection, it is not likely that much would be gained by extending hypothermia therapy further. Nonetheless, more protracted cooling may be beneficial especially in models of ICH that result in a significant amount of delayed edema and raised ICP.

The striatum is a difficult structure to protect after either global or focal ischemia, likely due, in part, to vascular differences and intrinsic differences in cell types (e.g., vs. cortex or CA1). In fact, several studies of hypothermia find better protection in cortex than striatum[18, 19, 37, 38]. For instance, hypothermia delayed for 1 hour after a focal ischemic insult, produced by temporary suture occlusion of the middle cerebral artery, resulted in a substantial reduction in cortical injury but only a trivial amount of striatum was protected[18]. Thus, it is possible that hemorrhagic insults in other structures, such as cortex, might be more amenable to hypothermia therapy.

A final consideration is that hypothermia (and other therapies) sometimes provides only transient benefit after cerebral insults. For example, after global ischemia brief hypothermia (e.g., 3 hr) will reduce CA1 sector cell death for several days only[14, 39]. Whereas prolonged hypothermia, as presently used, results in persistent benefit after global and focal ischemia[14-18] it may only transiently (e.g., by day 7) attenuate

histological damage and behavioral impairments after ICH.

The failure to detect deficits in the horizontal ladder task is in contrast to ICH-induced beam-walking deficits observed in other studies (e.g., [28-30]). Several explanations seem plausible. First, the tests are not identical and walking a beam could more difficult than traversing perpendicularly placed bars. Second, most studies utilizing the beam-walking test used it as a part of a series of neurological tests and report only the total score. Thus, the beam-walking task may not yield large deficits in those studies. Third, many studies of focal and hemorrhagic stroke use neurological test batteries during the first week after the insult. Subsequent to this time deficits on these tests largely resolve[29, 30, 40]. We did not perform neurological testing during the first week after ICH because half of our animals were being cooled up to and including the third day and we allowed several more days to recover (e.g. weight) after this therapy. Finally, there may be significant differences among these hemorrhage studies in location and size of lesion, which would certainly affect the magnitude and persistence of functional deficits. Thus, a larger lesion or a more laterally placed striatal lesion[41] may have resulted in greater deficits and may account for beam-walking deficits in other studies. Indeed a more recent hemorrhagic stroke study in our lab[42], which used a larger and more lateral striatal hemorrhagic lesion, did find significant and persistent deficits on the horizontal ladder task.

The cylinder task has not been used previously for striatal ICH. However, it is sensitive to focal ischemic injury[43, 44] and 6-hydroxydopamine lesions[35]. The likely reason we did not observe a significant (there were trends) deficit is that our insult was relatively mild, the lesion too medial, or that there was sufficient recovery (e.g., by

resolution of edema) by the time the first test was administered. For example, untreated ICH rats were observed to have their contralateral forelimb in a retracted posture in the days following ICH induction and they often did not use this paw when making contact with the wall of their home cage.

There are a variety of histopathological assessment methods and little consensus (e.g., number of sections to take, staining methods, etc.) on appropriate ways to determine the volume of brain injury. We found that the simple area assessment of the coronal section with the largest lesion was representative of the volume of tissue lost when many sections were obtained and a volume was determined. However, the collagenase injection did result in prominent ventricular enlargement in sections anterior, through, and posterior to the hemorrhagic lesion; an effect that is likely due to dendritic and axonal injury. Accordingly, it is important to consider these sections in any measure of lesion volume especially in cases of cytoprotection, which might differentially affect ventriculomegaly. Our findings suggest that a dozen equally spaced sections can be sufficient. Prolonged hypothermia did not affect ventriculomegaly, which is in line with the behavioral data showing absence of a beneficial effect. Nonetheless, it is possible that some beneficial (or detrimental) effects went unnoticed (e.g., axonal injury). Further study is needed with more sensitive functional tests and histological methods, especially given the positive finding of Kawai et al.[26].

In summary, delayed and prolonged hypothermia lessened neither subcortical lesion volume nor skilled reaching deficits in a collagenase model of ICH in the rat. Although unexpected, these results are consistent with the lack of substantial structural protection or functional benefit (in the Montoya staircase test) with other therapies (e.g.

surgical aspiration, anti-inflammatory drugs, or free-radical scavengers) using this ICH model. While we do not exclude other possible beneficial effects of hypothermia (e.g., ICP reduction), tissue that is quickly lost following ICH will not likely be salvaged.

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Figure 2-1: Core temperature (°C) averaged every 30 minutes (sampled every 30 seconds) starting after end of saline (SHAM and SHAM+HYPO) or collagenase infusion (ICH and ICH+HYPO). See Materials and Methods for further details. There were no differences among groups after 96 hr (data not shown).

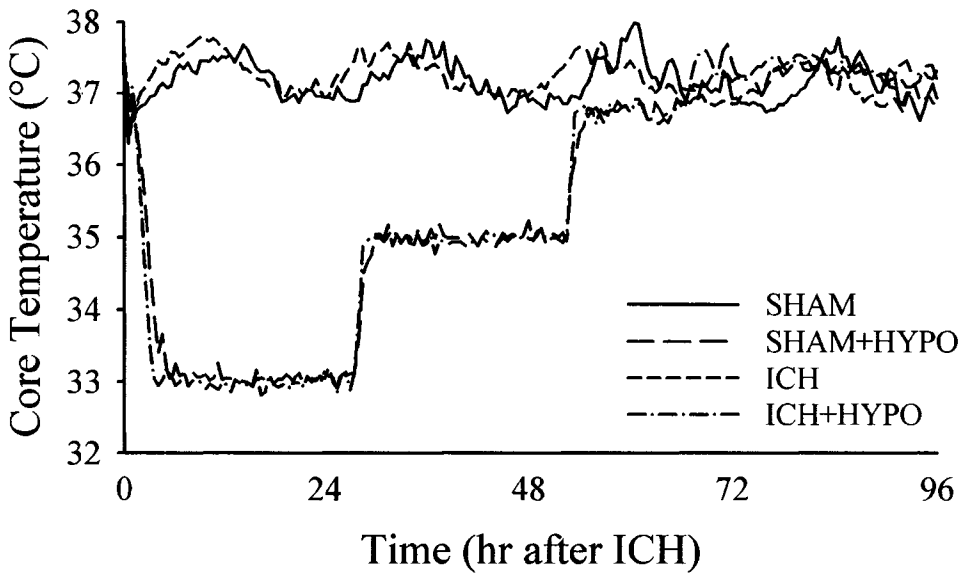


Figure 2-2: Performance (% baseline ± SEM) in the Montoya staircase test given on days 21 to 25 post-ICH or SHAM surgery for the ipsilateral (A) and contralateral (B) forelimb. Both ICH and ICH+HYPO groups were significantly impaired (vs. SHAMs) with the contralateral limb, but were not significantly different from each other.

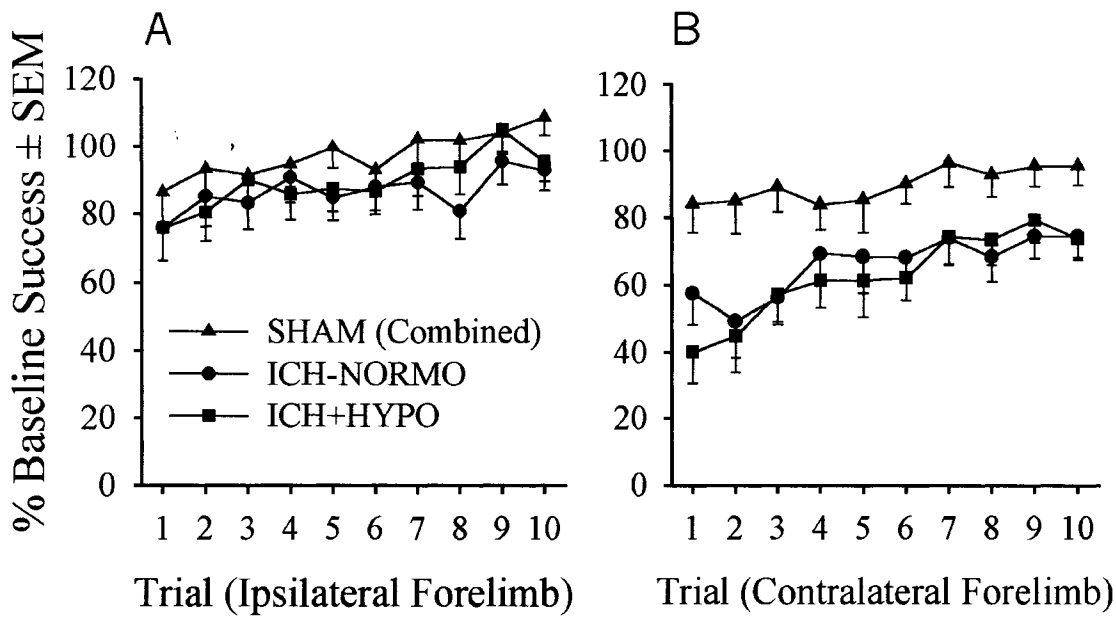


Figure 2-3: Performance (failure rate – %) with the contralateral forelimb in traversing the horizontal ladder on days 7, 14, 21 and 28 post-surgery. This test was not sufficiently sensitive to the ICH lesion although there was a trend towards impairment with the contralateral forelimb.

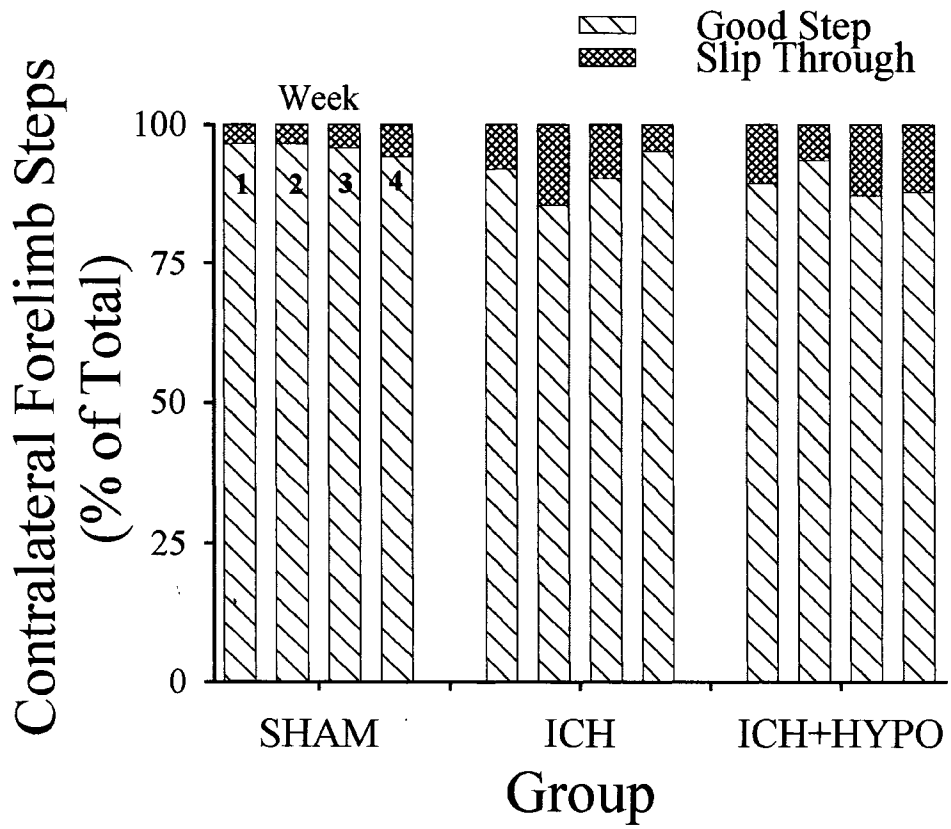


Figure 2-4: Percentage of paw placements on the wall in the cylinder test, which was given at 7, 14, 21 and 28 days post-surgery. There were no significant differences among the groups. Trends for push-off and landing were similar (data not shown).

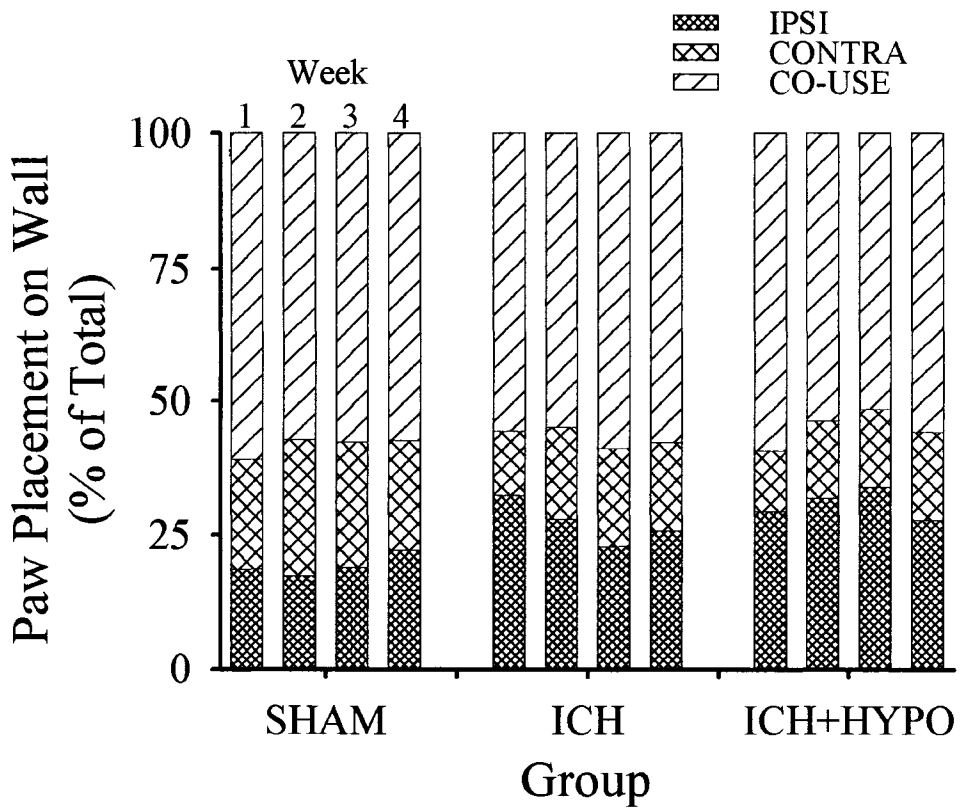
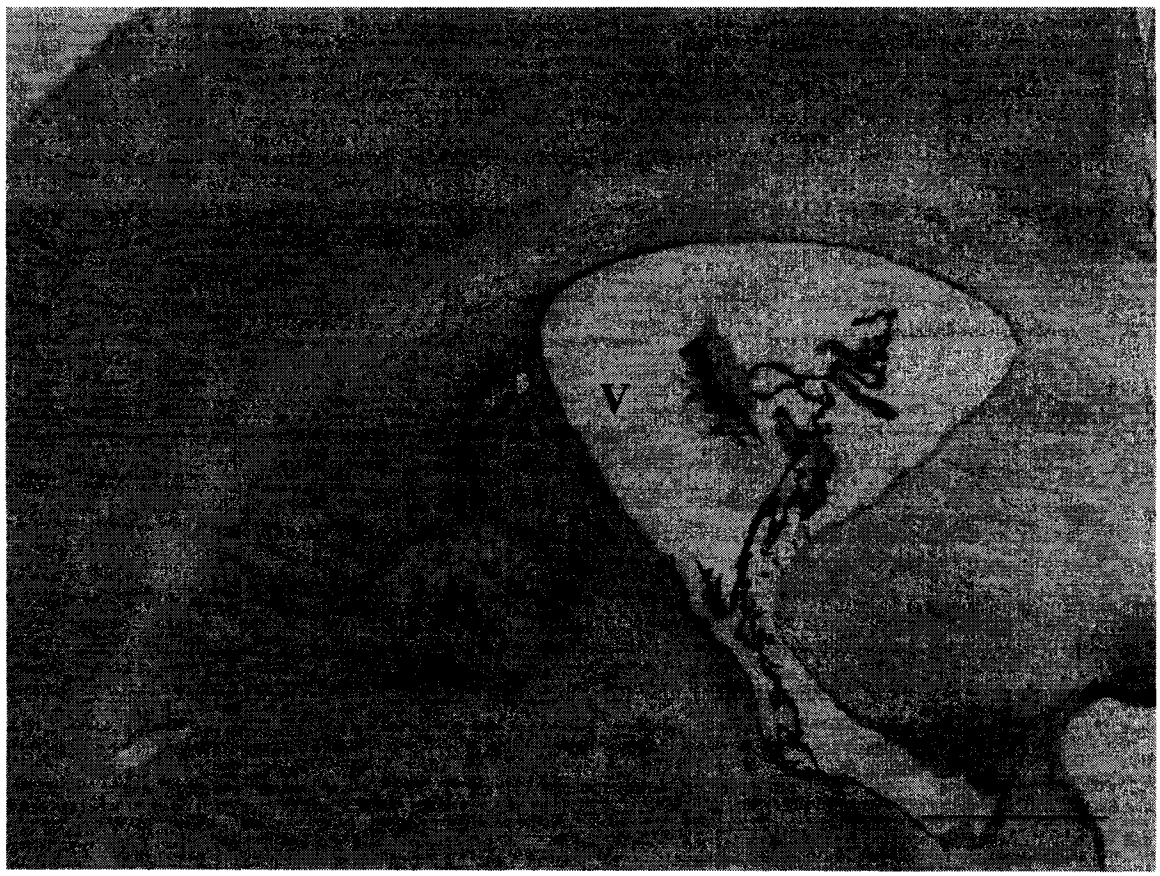


Figure 2-5: Photomicrograph illustrating the extent of brain injury following infusion of collagenase. The hematoma (H) was located primarily in the striatum but sometimes affected the globus pallidus, thalamus and the internal capsule. The lesion included a rim of degenerating tissue (arrow), which was likely inflammatory cells (e.g., microglia). Ventricular enlargement (V) was common. (Bar = 1 mm).



Chapter 3: Delayed and prolonged hypothermia improves outcome after intracerebral hemorrhage in rats

A version of this chapter has been published. MacLellan, C.L., Girgis, J., and Colbourne, F. 2004 *Journal of Cerebral Blood Flow and Metabolism*, 24: 432-440.

3-1 Introduction

Intracerebral hemorrhage (ICH) occurs when a blood vessel ruptures, releasing blood into surrounding brain tissue. ICH commonly occurs in the striatum, pons, thalamus and cerebellum, and accounts for approximately 15% of strokes in Western cultures[1]. Mortality is ~50% at 30 days and only 10% of survivors live completely independent[2]. Neurological impairments result from direct tissue destruction, surrounding tissue compression, cerebral edema, and reduction of cerebral blood flow[3]. Unfortunately, therapies aimed at treating these perturbations have not substantially improved outcome in patients. Several putative cytoprotectants have been tested in rodents including free-radical scavengers[4, 5], anti-inflammatory agents[6-9], thrombin inhibitors[10], anti-apoptotic agents[11], aspiration of the hematoma[12], and hypothermia[13, 14]. Although several of these therapies persistently reduce gross neurological impairments[4-6, 8], such as spontaneous rotation, none have improved performance in more demanding functional tests (e.g., skilled reaching) or provided substantial long-term histological protection. Long-term assessment is necessary since treatments may simply delay and not stop injury[15, 16].

Prolonged post-ischemic hypothermia improves functional recovery and reduces injury after global[17-19] and focal ischemia[20-22] in rodents to an extent that is arguably unsurpassed by pharmacological treatments. Hypothermia is a safe and feasible treatment for stroke[23-25] and global ischemia[26] in humans. Notably, recent clinical trials report improved outcome when prolonged hypothermia was induced after cardiac arrest [26, 27]. Because prolonged hypothermia is remarkably efficacious after global and focal ischemia, it might provide benefit after hemorrhagic stroke. Hypothermia may reduce secondary consequences of ICH such as ischemia in the peri-hematoma region,

inflammation, oxidative damage, edema, blood-brain barrier disruption and raised intracranial pressure. For example, hypothermia reduces edema and blood-brain barrier permeability after focal cerebral ischemia[28]. Furthermore, prolonged hypothermia (24 h) reduced vasogenic edema formation following intrastriatal thrombin injection in rats[13, 29]. Unexpectedly, however, when induced 1 hr after infusion of bacterial collagenase into rat striatum, prolonged (2 days) hypothermia failed to reduce volume of tissue lost or behavioral deficits[14].

We hypothesized that although hypothermia effectively treats components of hemorrhagic injury, failure to find protection in our previous study[14] may be due to deleterious physiological consequences of the early phase of the prolonged hypothermia treatment. For example, cooling conscious rats with fans and water misters may cause acute elevations in systemic blood pressure that promote further bleeding after cerebral hemorrhage in rats[30]. Furthermore, hypothermia is well known to inhibit enzymatic reactions of the coagulation cascade, causing abnormalities that exacerbate bleeding[31, 32]. Such adverse effects of hypothermia likely contribute to increased insult severity and may counteract the beneficial effects of prolonged cooling before the ICH has clotted.

Infusion of bacterial collagenase into the striatum disrupts vasculature and causes bleeding in surrounding brain tissue[33]. The progression of injury[34] and concomitant behavioural deficits[5, 35] are well characterized in this model of ICH. Whereas some behavioral effects (e.g., spontaneous rotation) largely recover over weeks, other functional deficits are long-lasting such as: contralateral forelimb stepping errors while walking across a beam or horizontal ladder, deficits in skilled reaching and diminished spontaneous use of the contralateral forelimb during exploration. Therefore, we used this

model and an appropriate array of functional tests to assess the efficacy of various mild hypothermia treatments induced 1 – 12 h after ICH. We hypothesized that cooling rats soon after ICH, during active bleeding, would exacerbate injury, whereas cooling many hours after injury would improve outcome. Thus, prolonged hypothermia delayed for 12 h after ICH was expected to be most beneficial, and early, long-lasting hypothermia was expected to provide no benefit. Finally, we hypothesized that early and brief hypothermia would be deleterious since it might aggravate bleeding and / or the lesion size but then is not long enough to protect against the delayed consequences of the ICH.

3-2 Materials and Methods

3-2a Animals

In total, 214 male Sprague Dawley rats (Ellerslie, Edmonton, Alberta, Canada) were used in this study. Rats weighed between 375 – 450 g (~16 weeks old) on the day of ICH. All procedures used were in accordance with the Canadian Council on Animal Care guidelines, and were approved by the Biological Sciences Animal Policy and Welfare Committee at the University of Alberta.

3-2b Montoya Staircase Training

Rats were food deprived to 90% of their free-feeding weight over 3 days and weighed daily to ensure stability at 90%. They were trained in the staircase test, which is a measure of independent forelimb reaching ability[36], over 15 days for two 15-min trials per day separated by 4 – 5 h, 5 days per week[20]. Fifteen rats were excluded because they failed to reach the criterion of 9 pellets (45 mg each; Bio-Serv, Frenchtown, NJ, USA) per side out of a possible 21 by the last three consecutive days during training.

3-2c Core Temperature Telemetry Probe Implantation

Seven days following the last staircase training session, rats were briefly (~10 min) anesthetized with isoflurane (4% induction; 1.5 – 2% maintenance) in 70% N_2O, 30% O_2. Core telemetry temperature probes (model TA10TA-F40; Transoma Medical, St. Paul, MN, USA) were implanted into the peritoneal cavity[37]. The wound was infiltrated with a local anesthetic (Marcaine; Sanofi Canada, Markham, ONT, Canada) and sutured closed. Rats were then housed individually on receivers (RPC-1; Transoma Medical) interfaced to a computer running telemetry software (A.R.T. 2.2; Transoma Medical) that sampled temperature every 30 s. Data from the complete day prior to ICH surgery served as a baseline.

3-2d Intracerebral Hemorrhage

Rats were anesthetized with isoflurane (1.5 – 2% maintenance) and placed in a stereotaxic frame. Throughout surgery (~30 min) core temperature was maintained near normothermia (37 °C) with a rectal temperature probe and heating blanket. Under aseptic conditions a midline scalp incision was made and a hole was drilled in the skull 3 mm lateral (right) and 0.2 mm anterior to Bregma. A 26-gauge needle (Hamilton syringe, Hamilton, Reno, NV, USA) was lowered 5.5 mm below the surface of the skull and 1.0 μL of sterile saline containing 0.2 U of bacterial collagenase (Type IV-S, Sigma, Oakville, ONT, Canada) was infused into the striatum over 5 min to create an ICH[14, 35]. The needle remained in place for an additional 5 min following infusion. A metal screw (model MX-080-2, Small Parts, Miami Lakes, FL, USA) was inserted into the thickness of the skull bone and the scalp wound was treated with Marcaine and closed with staples. In order to minimize invasiveness and duration of anesthetic, rats were not intubated

during surgery. Rats were randomly assigned to treatment condition and the abdomen and back were shaved to facilitate subsequent hypothermia treatment or to prevent knowledge of group identity in all rats.

3-2e Post-surgery Temperature Control

All animals were maintained near normothermia for one hour following infusion of bacterial collagenase. Some rats were kept above 37°C for an additional 11 h and were then allowed to regulate their own temperature (NORMO, N = 17). Other rats were slowly cooled beginning 1 h after infusion, by a rate of 1°C per 30 min to a core temperature of 33°C and maintained at this level for 24 h. These rats were then slowly warmed (1°C / 30 min) to 35°C and kept at that temperature for an additional 24 h before slow warming (HYP-1, N = 18). In some rats, the hypothermia treatment (as above) was initiated 6 h (HYP-6, N = 15) or 12 h (HYP-12, N = 17) after ICH induction. Other rats (BRIEF, N = 17) were cooled to 33°C starting 1 h after ICH, but maintained at this level for only 7 h before being heated to normothermia. Temperature was precisely (± 0.3°C) regulated by a servo-controlled system that used fans and fine water misters for cooling and infrared lamps for warming[37]. Temperature was monitored in all animals for seven days after surgery before telemetry probes were quickly removed under isoflurane anesthesia. One additional rat was excluded due to technical problems during surgery. An additional nine rats were excluded due to aberrant lesion placement. There was no mortality due to the ICH or treatment procedures in this part of the study.

3-2f Behavioral Testing

On days 7, 14, 21, and 28 the forelimb use asymmetry test (cylinder test)[35, 38] and the horizontal-ladder walking test[35, 39] were used to assess functional outcome. Rats were also tested in the staircase test on days 21 – 25.

Horizontal Ladder Walking Test

Each testing session was videotaped and consisted of three consecutive trials of rats walking across a horizontal ladder with variably spaced bars ranging from 1 to 3 cm. For each forelimb, the error rate (limb slips completely through the bars) made while traversing a 0.5 m segment of the ladder over three trials was determined.

Forelimb Use Asymmetry Test

Rats were placed in a transparent cylinder (45 cm in height and 33 cm in diameter) for 5 min and allowed to spontaneously explore the cylinder. A video camera set up below the cylinder recorded all forelimb placements on the wall, which were subsequently analyzed according to our previous work[35]. Briefly, we counted independent and initial forelimb use (exploration of wall after rearing) for contacting the cylinder wall during each test session. The percent of contralateral and ipsilateral forelimb use when exploring the cylinder walls was analyzed as [(number of contacts with contralateral limb / ipsilateral + contralateral limb use) × 100].

Staircase Test

Rats were food-deprived to 90% of their free-feeding weight 5 days prior to staircase testing. This consisted of 10 trials (two 15 min trials per day, separated by 4 – 5 h) on days 21 – 25 following ICH. The total number of pellets retrieved on each side was

recorded for each trial, and performance was expressed as a percent of baseline (average of the last 10 training trials when performance reaches asymptotic level).

3-2g Histopathology

Thirty days after ICH, rats were euthanized with an overdose of Somnotol (80 mg/kg i.p.) and were transcardially perfused with 0.9% saline and then 10% neutral buffered formalin. Forty-μm coronal sections were taken with a cryostat every 400 μm starting at +1.7 mm to Bregma and extending back to –4.8 mm to Bregma. Sections were stained with haematoxylin and eosin and using Scion Image J 4.0 (Scion Corporation, Frederick, MD, USA) the volume of lesion plus atrophy (e.g. ventricular enlargement) was quantified and expressed as follows[14, 35]:

Volume of tissue lost = remaining volume of normal hemisphere – remaining volume of injured hemisphere.

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.

The brains of four additional rats, which were only subjected to an ICH, were grossly dissected and visually examined at 6 hours following collagenase injection. This was to determine whether the ICH had fully clotted at that time.

3-2h Brain Water Content

Brain water content was determined in nine un-operated rats, which served as a control group. An additional 44 rats were used to assess the effects of an ICH and

hypothermia treatment on edema formation. The 44 rats were implanted with core temperature telemetry probes and three days later subjected to an ICH as described above. All rats were kept at normothermia for 12 h after surgery. Half of these then regulated their own temperature whereas the others received hypothermia treatment as for the HYP-12 group. Fresh brain tissue was removed at either two or three days post-ICH. Using a brain matrix (Ted Pella Inc, Redding, CA, USA) a 4 mm section (2 mm anterior and 2 mm posterior to needle injection site) was obtained and divided into each hemisphere. The weight of each fresh block was determined (i.e., wet weight). Brain blocks were placed in an oven (100°C for 24 h) and then re-weighed (i.e., dry weight). The water content of each block was determined as follows[40]:

Brain water content = [(Wet weight – dry weight) / wet weight] × 100.

3-2i Blood Pressure

In order to characterize acute changes in blood pressure after ICH, eight rats were implanted with a telemetry probe that measures temperature and blood pressure simultaneously (model TML2-C50-PXT; Transoma Medical). Three days later, rats were subjected to ICH and were maintained at normothermia for 12 h (NORMO; N = 4) or they received a brief hypothermia treatment (BRIEF; 33°C for 7 h, induced 1 h after ICH; N = 4). Temperature and blood pressure were sampled every 30 s and the complete day prior to surgery served as a baseline. These rats were not part of the behavioral or histological analysis.

3-2j Intracerebral Blood Volume

A modified spectrophotometric assay[41] was used to determine the volume of blood (hemoglobin) in the brain following ICH. Briefly, rats were overdosed with

Somnotol (80 mg/kg i.p.) and transcardially perfused with 100 mL of 0.9% saline to remove blood from the vasculature. The brain was then extracted and distilled water was added to it to reach a total volume of 3 mL. This solution was homogenized for 30 sec (Brinkman Instruments, ON, Canada) and then centrifuged (13,000 RPM for 30 min; model CR20B2, Hitachi, Japan). Several (minimum of 3) samples of the supernatant (100 μL) were collected and reacted with Drabkin's reagent (400 μL; Sigma) at room temperature for 15 min. Finally, absorbance readings at 540 nm (corrected for the background reading) were taken in a spectrophotometer (model DU-8, Beckman Coulter Ltd., London, UK) from several samples per rat and averaged. First, we generated a curve using known quantities of blood (0, 2, 4, 8, 16, 32, 64, 128 μL), which were obtained from a cardiac puncture, added to saline perfused normal rat brains (N = 11). Using the equation of the best fitting linear regression line from this data we determined the volume of blood remaining in other rats based upon absorbance readings. Thus, additional rats, which survived for 12 h post-ICH, were subjected to an ICH with (N = 13) or without (N = 15) the BRIEF hypothermia treatment. One additional rat died in the BRIEF group and was not included in the analysis. These rats were previously implanted with core temperature probes as described earlier. They were not part of the behavioral or histological volumetric analysis.

3-2k Data Analysis

All data are expressed as a mean ± SEM and were analyzed with ANOVA and by planned comparisons (SPSS, v. 11.0).

3-3 Results

3-3a Temperature

Baseline core temperature collected on the day prior to ICH surgery was similar among NORMO, BRIEF, HYP-1, HYP-6 and HYP-12 groups (overall mean = 37.14 °C ± 0.02; data not shown). Similarly, temperature during the 1 h after collagenase injection was similar among groups (overall mean = 37.12 °C ± 0.03; Fig. 1). Post surgical temperature was regulated as desired (see Methods). However, for 12 h after surgery the HYP-12 group was slightly (37.46 °C vs. 37.10°C), and significantly ($p < 0.001$) warmer than the NORMO group.

3-3b Body Weight

Body weight averaged 90.3% of baseline (± 0.83%) during staircase training. Body weight was similar among groups ($p = 0.324$) on the day of ICH (overall average = 418 g ± 2.01). Postoperative weight (Fig. 2) quickly returned to normal in the NORMO group and more gradually returned to normal in the hypothermia-treated groups. All groups were above baseline by day 14.

3-3c Staircase Testing

All groups performed similarly during training (data not shown). The test data were analyzed as a percentage of this baseline performance (last 10 trials). The ICH procedure (NORMO) resulted in a substantial reaching impairment with the contralateral limb (63.16% of baseline; $p = 0.001$; see Fig. 3). The ipsilateral forelimb success was not significantly lessened in the NORMO group (85.48 %; $p = 0.118$). Only the HYP-12 group appeared to be improved by hypothermia treatment. However, planned comparisons (HYP-12 vs. NORMO) showed no significant effect with either the

contralateral (81.27 % of baseline; $p = 0.123$) or ipsilateral forelimb (101.47% baseline; $p = 0.060$).

3-3d Horizontal Ladder Walking Test

The NORMO group had a mean error rate of 9.76% with the contralateral forelimb (Fig. 4). The HYP-1 and HYP-12 groups had significantly lower error rates at, on average, 5.39% ($p = 0.012$ vs. NORMO) and 6.14%, respectively ($p = 0.039$). The ipsilateral forelimb error rate did not differ significantly among groups (data not shown; $p = 0.172$).

3-3e Limb-use Asymmetry Test

There was a significant Group main effect for independent use of the contralateral limb ($p = 0.005$; Fig. 5). The BRIEF ($p = 0.021$), HYP-1 ($p = 0.001$) and HYP-12 ($p = 0.003$) groups were significantly improved overall (i.e., they used their contralateral forelimb more frequently than NORMO rats).

3-3f Composite Behavioral Score

All rats that had undergone behavioral testing (N = 85) were ranked from best to worst on their average performance (across trials) for each of the three behavioral tests. These three ranks were then averaged and then analyzed by ANOVA. The purpose of this procedure was to assess overall performance, and with this analysis only the HYP-1 (mean rank = 36.71 ± 3.98; $p = 0.040$) and HYP-12 (34.71 ± 3.79; $p = 0.020$) groups were significantly better (i.e., better performance) than the NORMO group (50.17 ± 4.53).

3-3g Histopathology

The lesion volumes at 30 days post-ICH are given in Fig. 6A. Untreated NORMO rats sustained damage primarily to the striatum, but also to the internal capsule, globus pallidus, thalamus and corpus callosum (Fig. 6B). Ventricular dilation was common ipsilateral to the lesion, but frequently occurred in the contralateral hemisphere as well. Prolonged hypothermia delayed 12 h after ICH reduced the volume of injury by 30.88% ($p = 0.047$ vs. NORMO). The HYP-1 ($p = 0.345$), HYP-6 ($p = 0.810$) and BRIEF groups were not significantly different than the NORMO group although there was a trend ($p = 0.063$) towards a larger lesion (by 28.46%) in the BRIEF group.

A visual inspection of several brains at 6 h post ICH indicated that the blood released into the brain had yet to be fully clotted, but was nearly so. The area of blood released into the brain also appeared to exceed that which was eventually destroyed (Fig. 6B).

All three behavioral tests significantly, although modestly, correlated with the volume of tissue lost when all groups were considered in the analysis. The Pearson r values were 0.26 ($p = 0.008$), -0.244 ($p = 0.012$) and -0.466 ($p < 0.001$) in the ladder, cylinder and staircase tests, respectively. Thus, there were greater functional deficits in rats with larger lesions.

3-3h Brain Water Content

The water content (one hemisphere block) in un-operated controls was 60.57% ± 1.63. This significantly increased to 65.50% ± 1.97 ($p = 0.050$ vs. control) and 66.09% ± 1.72 ($p = 0.026$) at 2 and 3 days, respectively, after untreated ICH (lesioned hemisphere). Brain water content was not significantly reduced by the HYP-12 treatment on either day

2 (66.25% ± 1.29; $p = 0.754$ vs. NORMO) or day 3 (62.97% ± 1.47, $p = 0.189$). There were no significant differences among groups in the contralateral hemisphere (data not shown).

3-3i Blood Pressure

Baseline blood pressure, collected the complete day before surgery, was similar between groups (108.78 mmHg ± 3.30; $p = 0.367$). In order to determine if hypothermia significantly elevated blood pressure, we averaged data collected over the first 6 h after the induction of hypothermia. This period encompassed the maximal elevation in pressure apparent approximately 1 – 1.5 h after the induction of hypothermia (Fig. 7). Post-ICH pressure (107.51 mmHg ± 3.41) in normothermic ICH rats did not significantly differ ($p = 0.528$) from baseline. Brief hypothermia induced 1 h after ICH caused a significant and prolonged (at least six hours) blood pressure hypertension relative to untreated rats (118.76 mmHg ± 2.41; $p = 0.036$).

3-3j Intracerebral Blood Volume

Rats subjected to the BRIEF cooling treatment after ICH had an estimated blood volume of 79.2 μL ± 6.98 whereas normothermic ICH rats has a volume of 58.4 μL ± 5.29 ($p = 0.023$). Thus, the BRIEF treatment significantly increased bleeding by 35.6% at 12 h post-ICH.

3-4 Discussion

Prolonged and delayed (12 h) mild hypothermia reduced the ICH lesion volume and concomitant behavioral deficits after a moderate sized ICH in rats. To date this is the only cytoprotective therapy that persistently (i.e., 30 day survival time) reduces the volume of injury after ICH. Remarkably, the greatest histological protection occurred

with a treatment starting 12 h after the ICH whereas earlier treatments failed to lessen the volume of tissue lost. This contrasts with findings after both global and focal cerebral ischemia, where the greatest protection occurs when hypothermia begins as early as possible[18, 22, 42, 43]. Thus, the ideal starting time of hypothermia therapy in the clinic may critically depend upon the type of insult.

We suspected that side effects in the early phase of hypothermia treatment would negate the beneficial effects of the therapy after ICH resulting in no net protection. Indeed we previously observed[14] no histological protection with a 1 h delayed treatment and this was again found in the HYP-1 group. Furthermore, neither the BRIEF nor HYP-6 groups had histological protection. One possible side effect is coagulopathy (slowed clotting time), which is well documented during hypothermia[31, 32]. Our method of cooling using fans and water misters[37] is also stressful to conscious rats temporarily elevating blood pressure in cooled ICH rats (present study) as well as in normal rats (MacLellan, Wiltshire and Colbourne, unpublished data). Notably, acutely elevated blood pressure increases hemorrhage volume after needle biopsy in rats[30]. Therefore, both coagulopathy and hypertension likely exacerbated bleeding in our study with the BRIEF treatment and contributed to increased insult severity in treated ICH rats that were still actively bleeding (i.e., BRIEF, HYP-1 and HYP-6 groups). Finally, visual observations at 6 h after ICH indicated that the hematoma was not fully formed at that time in this model, which may account for the lack of structural protection in the BRIEF, HYP-1 and HYP-6 groups. Further experiments designed to determine whether these and other side effects of hypothermia exacerbates hemorrhagic injury were beyond the scope of this efficacy study but are planned (e.g., regulating blood pressure).

We suspect that hypothermia (even when delayed for 12 h) counteracts multiple mechanisms of hemorrhagic tissue damage (e.g., inflammation, oxidative stress, ischemia). Perhaps it is because hypothermia counters many injurious cascades that long-term histological and functional protection occurs, whereas most pharmacological agents that generally target one component of injury, such as oxidative damage[5], fail to provide histological benefit against hemorrhagic stroke. One prominent contributor to a poor outcome is edema, which hypothermia is well know to reduce[42, 43]. For instance, hypothermia reduces swelling after experimental focal ischemia[28, 44, 45] and intrastriatal thrombin injections[13, 29]. Thus, it was surprising that the reduction in edema (~3% at three days post-ICH) by hypothermia was not statistically significant in this study. Perhaps more animals were needed in each group or the sampling times (2 and 3 days post-ICH) were not ideal. However, pilot work in our lab indicates that edema is minimal at 1 and 4 days post-ICH. Finally, it is possible that hypothermia reduces edema but it rebounds during the re-warming phase, which is when we sampled.

One limitation of this study is that we did not determine the extent by which 12 h delayed hypothermia reduces striatal cell death. There was a significant reduction in the volume of tissue lost; however, it is also possible that hypothermia reduced injury outside of the primary lesion site (e.g. reduced dendritic atrophy of surrounding neurons and less axonal degeneration). Indeed, the volume of the tissue lost increases over days to weeks in this ICH model[34, 46] and this is unlikely to be due solely to continuing cell death. Thus, the long-term protection observed in the HYP-12 group could result from reduced cell death as well as limiting delayed degenerative events. These possibilities are not easily dissociated. Regardless, the primary purpose was to determine whether hypothermia

could persistently improve outcome, which it did. Future studies should determine whether protection is permanent (e.g., 6 month survival time). Cell counts should be used in those studies to determine the locus of protection.

There are several limitations with the collagenase ICH model to consider. Most importantly, the model we used, which resulted in greater injury than many studies, produced variably sized lesions that did not consistently affect one structure (e.g., lateral striatum). This variability is not surprising given the nature and severity of the insult. Such inherent variability causes more variable behavioral deficits (e.g., cylinder data) and contributes to the imperfect correlations between behavioral scores and lesion volume in this study, which is also common in the literature[47]. For instance, functional deficits on simple behavioural measures (e.g. neurological deficit score) often spontaneously recover in spite of substantial brain injury[48]. As well, several studies of hemorrhagic stroke demonstrate significant reductions in neurological deficits without histological protection[5, 6, 8]. In addition to variability in the size of a lesion one must consider that there are individual differences in brain organization, motivation, recovery mechanisms and the presence of undetected damage that all contribute to discordant histological and functional outcomes. Therefore, it is not surprising that we detected behavioural, but not histological improvement with the HYP-1 treatment whereas both were found in the HYP-12 group. We attempted to overcome these problems by using relatively large group sizes as well as multiple testing sessions and three sensorimotor tests. As well, we used a composite behavioral score. The use of both histological and multiple functional assessments are required in assessing the effectiveness of any therapy[47, 49].

An additional problem with this model was that the lesion sometimes affected the contralateral hemisphere (i.e., apparent bilateral atrophy). This effect appeared to be least common in the HYP-12 group. Accordingly, we may have underestimated the volume of lesion more so in the NORMO group and thereby underestimated the protective effect of the HYP-12 therapy. Unfortunately, there is no simple way to overcome this problem. An analysis of only the lesioned side does not correct for individual differences in brain size (i.e., compared to the contralateral hemisphere) or differences in coronal section selection.

A final consideration is whether prolonged hypothermia would similarly affect other models of hemorrhagic stroke (e.g., subarachnoid hemorrhage) or even alternative ways of modeling a striatal ICH (e.g., blood injection method). Unfortunately, neither the collagenase or blood injection model perfectly reflects the clinical situation. We predict that early hypothermia may provide greater benefit after direct intrastriatal autologous blood injection than after collagenase injection because in the latter there is more active bleeding that would presumably be increased by hypothermia. This hypothesis is currently being investigated and these issues must be resolved before hypothermia is applied to ICH patients. Finally, other insults that cause intracranial bleeding, notably traumatic brain injury (TBI), may not be similarly affected by hypothermia as it reduces, not increases, extravasated hemoglobin levels in a rat model of TBI[50]. This difference may relate to the time course, quantity or location of bleeding in TBI versus ICH as well as to method of cooling. Thus, the latter parameter will be investigated in ICH in our lab.

In summary, when delayed 12 h, prolonged mild hypothermia provides persistent histological and functional protection after striatal ICH in rats. Hypothermia is thus the

only treatment, at present, that persistently reduces injury and improves functional outcome after experimental ICH. While these findings suggest that clinical investigation is warranted, there are a number of issues that must be resolved first. Notably, in addition to identifying the optimal hypothermia profile (delay, depth and duration) and the mechanisms of action, investigators should study pharmacological means of cooling or alternatively to counteract known side effects (e.g., coagulopathy) of whole-body hypothermia during the treatment. A mechanistic understanding of hypothermia's beneficial and deleterious effects could lead the way in finding alternative, less risky, therapies. We also suggest that hypothermia be tested in larger gyrencephalic animal models of hemorrhagic stroke with more attention to clinically relevant issues (e.g., sex, age, co-morbidities such as hypertension)[49, 51].

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Figure 3-1: Core temperature (°C) for four days following ICH. Temperature from 5 – 7 days was similar (data not shown). See Methods for temperature manipulations. The temperature profiles (data now shown) of the rats used for collecting BP readings, edema measurements or estimating blood volume with spectrophotometry were similar to their respective groups shown here.

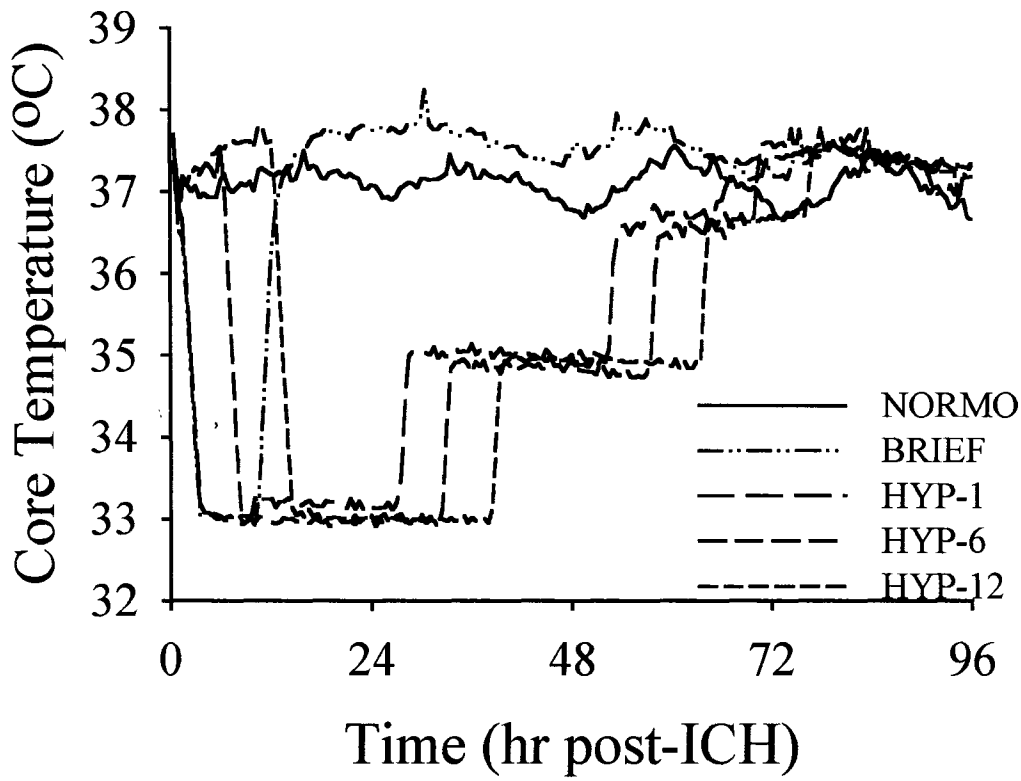


Figure 3-2: Postoperative weight (% baseline) on days 1 through 7 and day 14 post ICH. See Results for statistics.

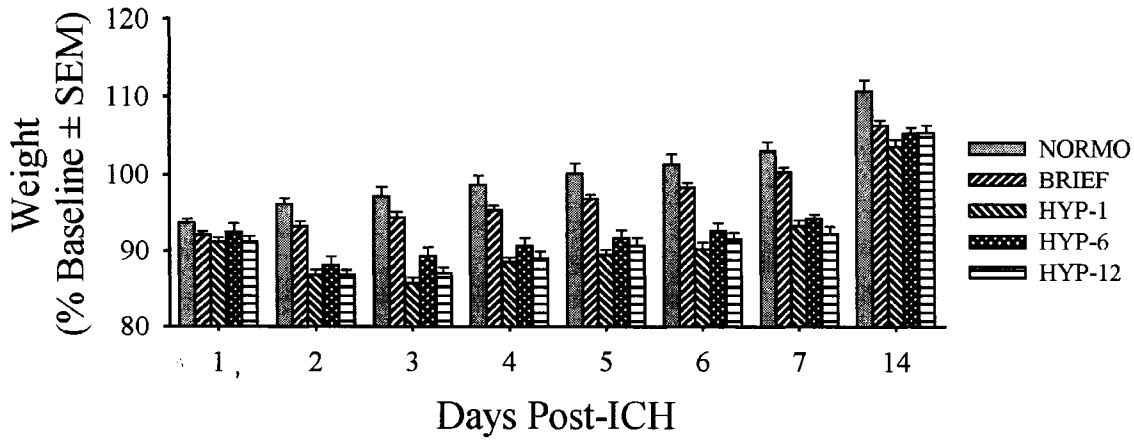


Figure 3-3: Success (% baseline) with the ipsilateral (top) and contralateral (bottom) forelimbs in the Montoya staircase apparatus over 10 test trials. See Results for statistics.

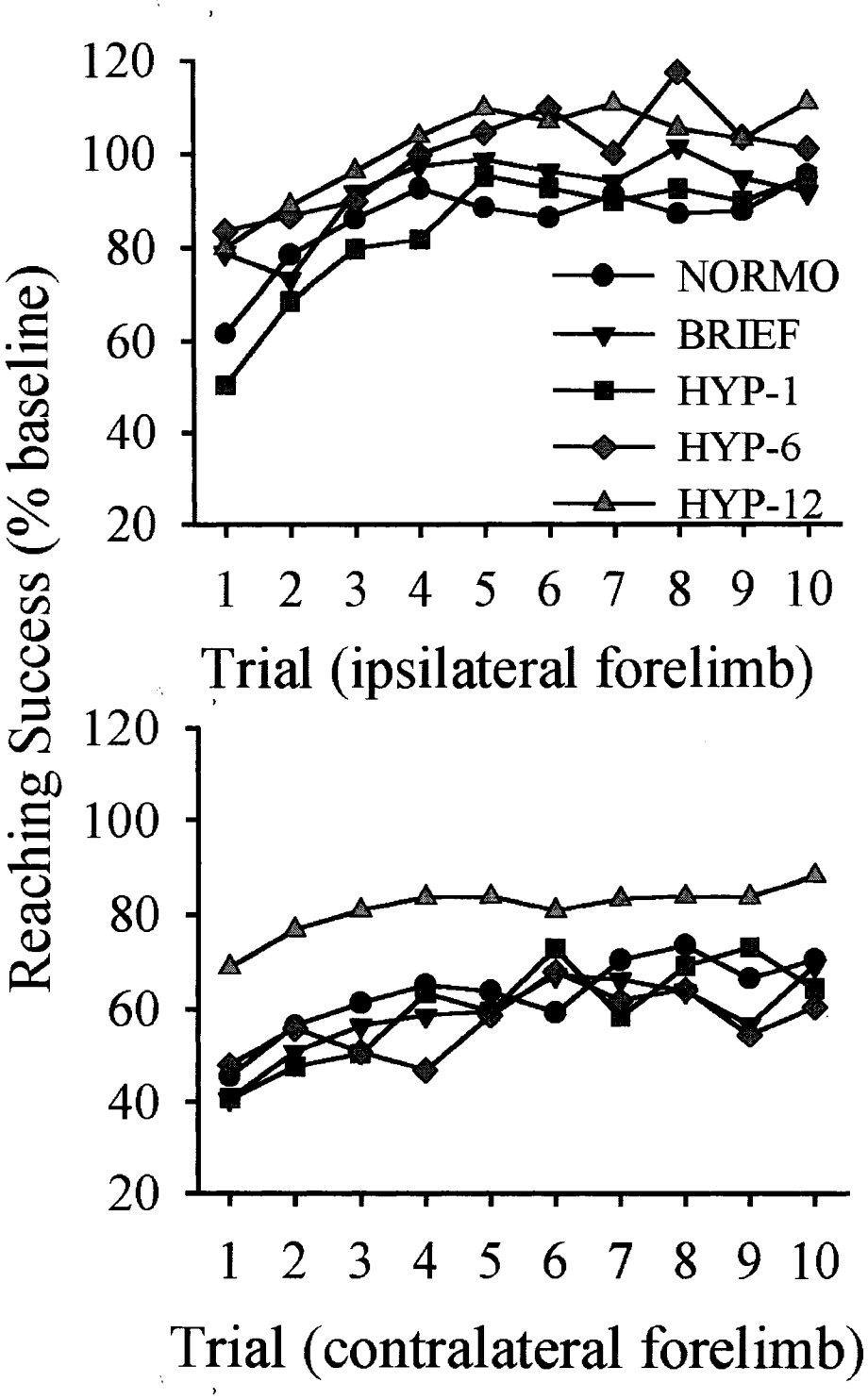


Figure 3-4: Error rate (% falls through bars; A) in the horizontal ladder test (B) with the contralateral forelimb at 7, 14, 21 and 28 days following ICH. See Results for statistics.

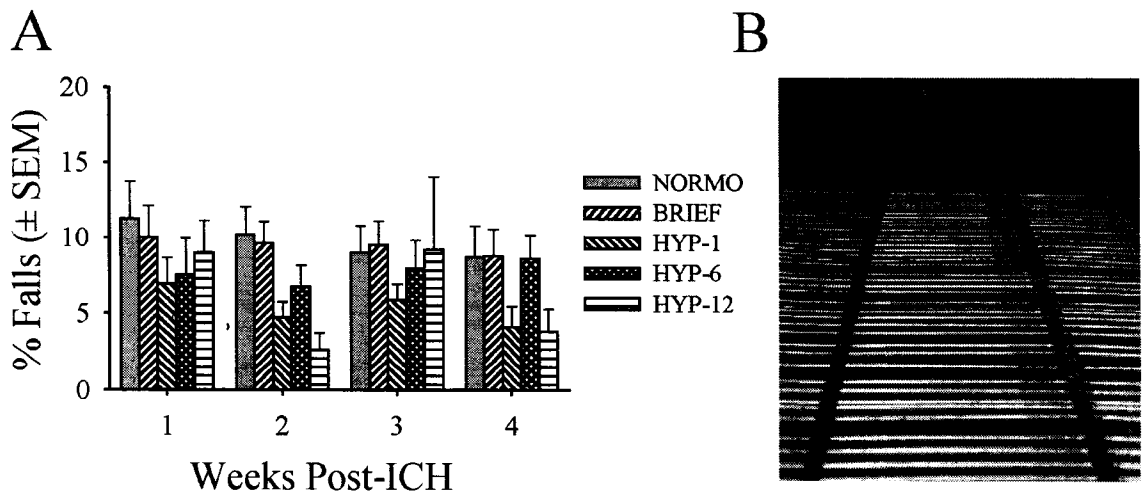


Figure 3-5: Spontaneous contralateral forelimb use [(number of contacts with contralateral limb / ipsilateral + contralateral limb use) × 100] in the cylinder test on days 7, 14, 21 and 28 after ICH.

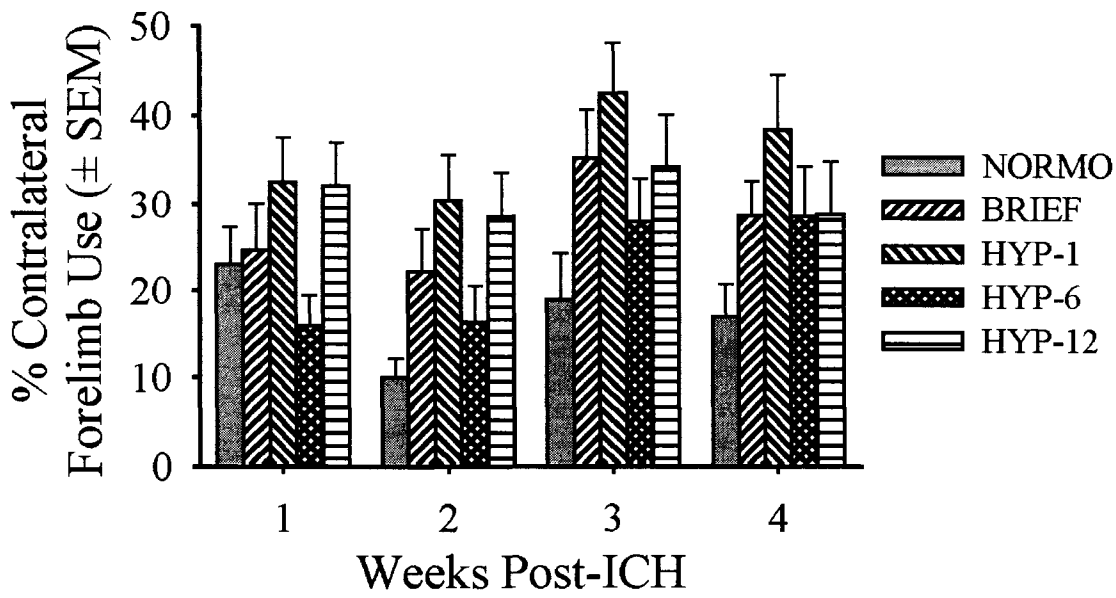


Figure 3-6: Lesion volume (mm^3; A) at 30 days post ICH. A typical lesion, illustrated in B, included substantial striatal injury, ventriculomegaly, along with damage to several other structures (e.g., thalamus, globus pallidus).

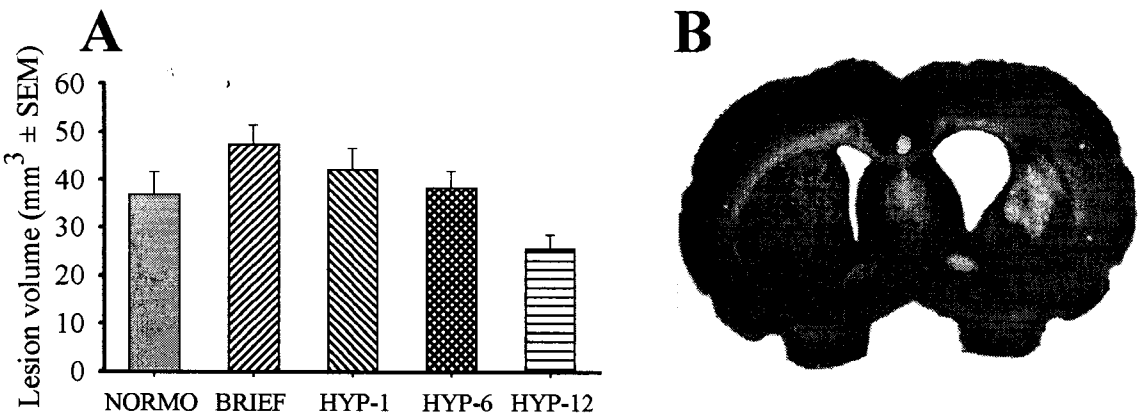
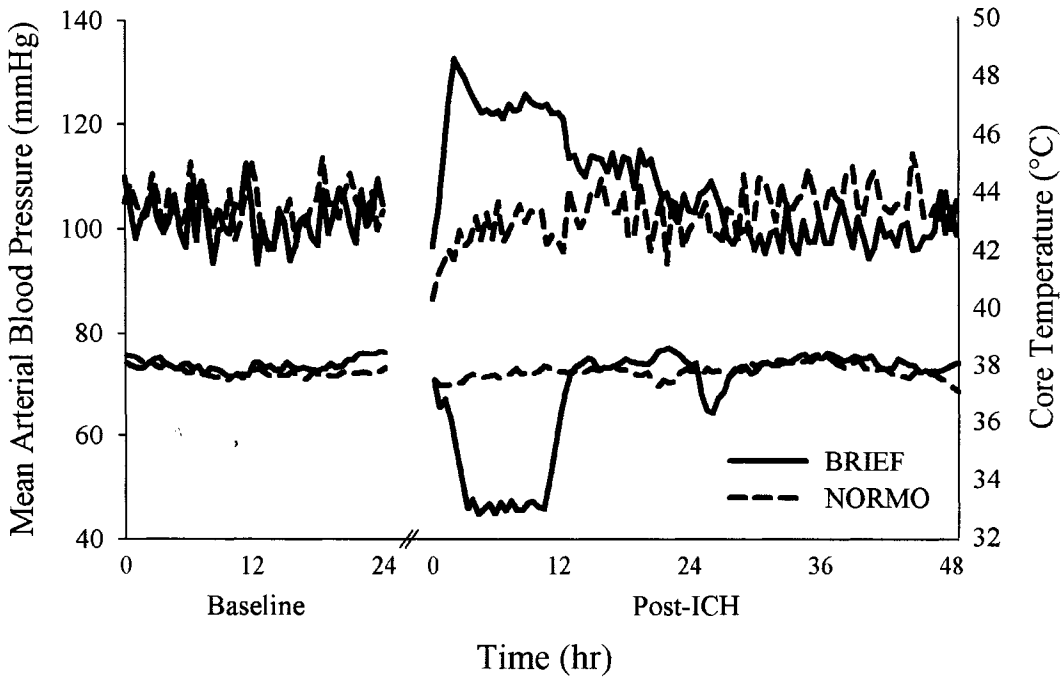


Figure 3-7: Mean arterial blood pressure (mmHg; left y-axis) and core temperature (°C; right y-axis) in two representative ICH rats (one NORMO and one BRIEF) during baseline and after ICH. Hypothermia caused a significant elevation in blood pressure. See Results for statistics.



Chapter 4: The influence of hypothermia on outcome after intracerebral hemorrhage in rats.

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4-1 Introduction

Spontaneous intracerebral hemorrhage (ICH) causes high mortality and poor recovery in survivors. Recombinant activating factor VII (rFVIIa) is currently the only drug that improves outcome for ICH patients[1]. However, it is limited to those who promptly receive attention and who undergo hematoma expansion. Thus, cytoprotective treatments are needed. In ICH, significant tissue damage occurs quickly due to space occupying effects and toxicity of the degrading hematoma. Secondary consequences of ICH, and ischemia, include inflammation[2], edema[3], and oxidative damage[4], which all contribute to cell death[5, 6].

Mild, prolonged hypothermia (HYPO) improves outcome in rodent models of global[7] and focal ischemia[8, 9]. Furthermore, HYPO can be safely applied to stroke victims and significantly benefits cardiac arrest patients[10]. Given the overlap in mechanisms contributing to injury after ischemia and ICH, and the fact that HYPO favorably affects deleterious processes common to both, it makes sense to test HYPO after ICH. Early work shows that HYPO reduces edema after intrastriatal thrombin injections[11] and after ICH[12, 13] in rats. Also, local HYPO reduces edema in a pig ICH model[14]. However, contrary to ischemia where earlier cooling is more efficacious[7, 9], HYPO initiated soon after collagenase-induced ICH, during active bleeding, increases hematoma size[15]. This effect is apparently due to side effects of HYPO (e.g., elevated blood pressure, coagulopathy), which would counteract beneficial effects of HYPO during the early post-ICH period. Not surprisingly then, HYPO delayed 12 hours after ICH improves recovery and lessens tissue loss as bleeding would not be aggravated at that time[15]. Accordingly, we hypothesized that early HYPO should improve outcome in the whole blood model of

ICH because bleeding is expected to end at or soon after infusion. Therefore, we assessed whether post-ICH HYPO affects bleeding, BBB permeability, edema, inflammation, neuronal degeneration, lesion size and behavioral recovery.

4-2 Materials and Methods

4-2a Animals

We used 254 male Sprague-Dawley rats (~16 weeks old; ~375 g). All procedures were approved by the University of Alberta and followed the Canadian Council on Animal Care guidelines.

4-2b General Procedures

Surgical procedures were performed aseptically under isoflurane (4% induction; 2% maintenance in 70% N_2O and 30% O_2).

Temperature Probe Implantation

A telemetry probe (TA10TA-F40; Transoma Medical, St. Paul, MN) was implanted into the peritoneum 3 days prior to ICH[15]. Core temperature was sampled every 30 seconds and the day prior to ICH served as a baseline. Brain temperature was not measured due to technical difficulty in securing a head cap while permitting striatal blood infusion.

Intracerebral Hemorrhage

Anesthetized animals were placed in a stereotaxic frame. A midline scalp incision was made and a burr hole was drilled 3.5 mm right of and at the AP level of Bregma. To create an ICH, 100 μL of autologous blood withdrawn from the tail was injected into the striatum (depth of 6.5 mm) over 10 minutes. After another 10 min the needle was slowly removed. A metal screw sealed the hole, and the scalp was closed followed by

application of Marcaine (Sanofi, Markham, ONT). During surgery (~ 45 minutes), core normothermia was maintained (i.e., 36.5 – 37.5°C). The abdomen and back were shaved in all animals to facilitate cooling in HYPO rats and to prevent our knowledge of group identity in NORMO rats. Rats were weighed daily after ICH up to seven days. In Experiment 2, which was taken to represent all studies, we measured mean arterial blood pressure (MABP; via tail artery) throughout surgery and arterial physiological measurements (pH, pCO_2, pO_2, Hb, glucose) before and after ICH. A small amount of heparinized saline was used to prevent clotting in the tail artery and catheter. This was not done in other studies.

Post-ICH Temperature

Rats were maintained near normothermia for 1 hour post-ICH. The NORMO group was regulated above 36.5°C for 48 hours. Others were slowly cooled at a rate of 2°C/hour to 33°C starting 1 (HYPO-1) or 4 hours (HYPO-4) after ICH and maintained at this level for 24 hours. Rats were warmed (2°C/hour) to 35°C for an additional 24 hours before rewarming to normothermia (Figure 1) as previously done[15]. Temperature was precisely servo-regulated using infrared lamps, fans and fine water misters.

4-2c Experiment 1: Hematoma Volume

Hematoma volume was measured in NORMO (n = 7 included) and HYPO-1 (n = 7) rats 12 hours after ICH using a spectrophotometric hemoglobin assay described elsewhere[15, 16].

4-2d Experiment 2: Blood Brain Barrier Disruption

We assessed Evan's blue extravasation (n = 20/group) 48 hours post-ICH. Evan's blue dye (Sigma; 2% in saline; 4 mL/kg) was injected intravenously. Two hours later, rats

were perfused with saline and each hemisphere was weighed, homogenized in saline, and centrifuged. The supernatant was incubated with 50% trichloroacetic acid, centrifuged, and absorbance was read on a spectrophotometer at 610 nm. Four un-operated rats served as a non-hematoma control. Thirteen additional rats were used to generate a standard curve using known amounts of dye (0.1 μL to 1.0 μL) added to un-operated control hemispheres. The amount of extravasated Evan's Blue dye was calculated from this curve.

4-2e Experiment 3: Brain Water Content

Brain water content was measured in six un-operated rats and at two days post-ICH (n = 10 in each of three groups). After decapitation (under anesthesia) the cerebellum and four mm thick sections of striatum and cortex of each hemisphere were weighed (wet weight), baked at 100°C for 24 hours, and reweighed (dry weight). Water content was determined by [(wet weight – dry weight)/wet weight] × 100.

4-2f Experiment 4: Assessment of Iron-Positive Cells, Neutrophils, and Degenerating Neurons

Four days after ICH, all groups (n = 10/group) were euthanized and brains were cut into 10 μm coronal frozen sections. Iron positive cells and neutrophils were stained using Perl's Prussian blue for ferric iron[6, 17] and Leder's stain[18] for chloracetate esterase activity (Sigma), respectively. Degenerating neurons were stained with Fluoro-Jade B (BioChemika)[19]. The total number of iron positive cells, neutrophils, and degenerating neurons was counted in the lesioned hemisphere at the level of maximum hematoma diameter.

4-2g Experiment 5: Long-term Outcome

Rats (n = 21/group) were trained on cylinder[20], horizontal ladder[21] and staircase tests[22]. These tests measure spontaneous forelimb usage, walking and skilled reaching ability, respectively, and are sensitive to striatal ICH[23, 24]. Baseline performance and training was done prior to core probe implantation[15]. Rats were evaluated on the ladder (% successful steps) and cylinder (asymmetry score; ipsi – contralateral touches) tests 7 and 30 days following ICH, and on the staircase from days 24 to 28.

Thirty days after ICH, rats were euthanized with pentobarbital (80 mg/kg) and perfused with saline then 10% formalin. Forty-μm coronal brain sections taken every 400 μm were stained with cresyl violet. Lesion volume (cellular debris, ventriculomegaly, and cavity) was manually determined using Scion Image J[15]:

Volume of tissue lost = remaining volume of normal hemisphere – remaining volumeof lesioned hemisphere.

Volume of a hemisphere = average (area of hemispheric coronal section – area of ventricle - area of damage) × section interval × number of sections.

4-2h Experiment 6: Short-term Outcome

NORMO and HYPO-1 treated rats (n = 8 each) survived for 7 days post-ICH. Functional outcome was assessed using the ladder and cylinder at 7 days and lesion volume was assessed.

4-2i Statistics

All procedures were done by experimenters blind to group identity. Using SPSS (v 12), data were analyzed with ANOVA and LSD post-hoc tests if needed. Data are presented as the mean ± S.E.M. A p value of < 0.05 was considered to be statistically

significant.

4-3 Results

In addition to the number of rats stated in each experiment, an additional 21 rats were excluded, of which 20 were excluded due to technical problems (e.g., computer crash during temperature regulation) and the other rat was euthanized 8 days post-ICH for failure to maintain normothermia.

4-3a Physiological Variables

Blood gases, Hb, pH, and glucose (Experiment 2) were in normal ranges and did not differ significantly among groups (Table 1). During surgery MABP was slightly but significantly higher in the HYPO-4 group than the NORMO and HYPO-1 groups. Baseline core temperature collected before ICH was similar among groups (36.76 ± 0.04°C; data not shown), and post-ICH temperature was regulated as desired. For instance, in Experiment 5 (Fig. 1) temperatures were on average 33.0 ± 0.01 °C and 34.9 ± 0.02 °C during the designated periods of 33 and 35°C cooling, respectively.

4-3b Experiment 1: Hematoma Volume

The hematoma volume was not significantly different in NORMO (79.2 ± 6.1 μL) and HYPO-1 groups (82.1 ± 8.9 μL).

4-3c Experiment 2: Evans Blue Extravasation

We detected a small amount of Evan's blue dye in the non-hematoma control brains (0.7 ± 0.2 μg dye/g tissue). Evans blue extravasation, corrected for this baseline reading, in the ipsilateral hemisphere was significantly reduced by HYPO-1 and HYPO-4 treatments (Fig. 2). Little extravasation occurred in the contralateral hemisphere, which was not significantly different among groups.

4-3d Experiment 3: Brain Water Content

Compared with un-operated controls (78.3 ± 0.1%), the NORMO rats had significantly increased brain water content in the damaged striatum (80.0 ± 0.2%), which was significantly reduced by HYPO-1 (79.4 ± 0.2%), but not HYPO-4 treatment (79.8 ± 0.3 %).

4-3e Experiment 4: Iron-Positive Cells, Neutrophils, and Degenerating Neurons

Neutrophils infiltrated the hematoma and surrounding tissue by four days (Fig. 3A) whereas iron-positive cells (likely activated microglia or macrophages) were in the surrounding tissue (Fig. 3B). Both hypothermia treatments significantly reduced neutrophil and iron-positive cell infiltration. The number of degenerating neurons was similar among groups (Fig. 3C).

4-3f Experiment 5: Long-term Outcome

Fifteen rats (~ 5/group) failed to retrieve at least 9 pellets per side on the last 3 days of training in the staircase[23], and were excluded from just this analysis. Analysis of contralateral forelimb success revealed a significant GROUP × TRIAL interaction. Upon further analysis the HYPO-1 group obtained significantly more pellets on the first day (vs. NORMO; Fig. 4A), but all groups performed similarly afterwards (i.e., full recovery). In the ladder-walking test, the percentage of successful steps made with the contralateral forelimb on days 7 or 30 was similar among groups (data not shown). The HYPO-1 group made more successful steps with the contralateral hind limb on day 7 (vs. NORMO and HYPO-4 groups; Fig. 4B), but not day 30. Limb use (asymmetry score) in the cylinder was equivalent in all groups on days 7 and 30 (Fig. 4C). After ICH, damage occurred primarily to the striatum and corpus callosum (Fig. 5B). Hypothermia

treatments did not affect the volume of tissue lost (Fig. 5A).

4-3g Experiment 6: Short-term Outcome

Behavioral impairments in the cylinder (19.5 ± 15.2 vs. 15.2 ± 7.0) and ladder (forelimb success: 84.8 ± 5.5% vs. 85.4 ± 3.9%; hind limb success: 84.1 ± 6.4% vs. 85.3 ± 6.1%) tests as well as lesion size (7 day survival; 30.0 ± 2.3 mm^3 vs. 34.8 ± 3.8mm^3) were not significantly different between NORMO and HYPO-1 groups, respectively.

4-4 Discussion

The present findings do not support the use of HYPO soon after ICH as our short- and long-term outcome studies largely found no benefit with either HYPO treatment. Nonetheless, HYPO significantly, but modestly reduced edema and substantially reduced BBB disruption and inflammation. Accordingly, reductions in edema, inflammation or BBB disruption may not necessarily translate into functional and histological improvements. Therefore, any putative therapy should be comprehensively assessed (e.g., recovery, injury, edema, etc.) prior to clinical investigation.[25]

As discussed, HYPO provides significant protection in models of global and focal cerebral ischemia. Conversely, hyperthermia aggravates ischemia[26]. Our present findings with HYPO and our recent findings with induced hyperthermia[17] suggest that ICH is less temperature sensitive than ischemia. In our hyperthermia study, forced elevations in temperature did not significantly worsen outcome after ICH. Interestingly, the clinical findings on ICH and hyperthermia are contentious.

There are several study limitations that warrant consideration. First, we did not determine whether HYPO affects edema, BBB disruption and inflammation at other times. Thus, HYPO may have postponed these processes as found in focal ischemia

where HYPO delayed inflammation[27]. If so, longer cooling may be more efficacious. Second, it appears that HYPO provides better protection in the collagenase model[15] than in the whole blood model. However, further study is needed to confirm this because we did not directly compare treatments (e.g., onset delay) in models matched for insult severity. For instance, our collagenase study found benefit with cooling delayed for 12 hours, which was not presently tested. Thus, it is possible that later cooling would provide benefit in the whole blood model. However, aside from HYPO aggravating bleeding in the collagenase model, there is no obvious mechanism as to why later cooling would provide better protection and this was not seen in comparing HYPO-1 and HYPO-4 groups. Third, we used heparinized saline to prevent clotting in the tail artery in Experiment 2, which was accessed for BP and blood gas analysis. While the rest of the studies did not use heparin, it is possible that the results of Experiment 2 are somehow affected compared to the other experiments. Fourth, the statistically significant reduction in edema must be considered modest at best. Therefore, the apparent lack of benefit of this edema reduction must be interpreted with caution. Indeed, we expect that greater reductions in edema would provide some benefit, although this should always be tested. Fifth, the HYPO-1 treatment improved hind limb success in the ladder at day 7 post-ICH in Experiment 5, but not 6. As there is no obvious explanation for this, it is possible that these hind limb findings in Experiment 5 are due to chance, especially considering that forelimb success was not different in either experiment, and generally, other tests showed no benefit. Finally, assessing functional outcome in the whole blood model was problematic because animals showed good recovery with each test. Thus, without substantial behavioral impairments, one cannot easily assess protective effects (i.e.,

ceiling effect). We attempted to overcome this by repeatedly using several tests sensitive to striatal injury[23].

In summary, early and prolonged HYPO reduced several consequences of ICH, but provided very little functional benefit and no discernable histological protection. Accordingly, further study is needed to improve HYPO before it is applied to ICH patients. Importantly, side effects of HYPO must be identified and countered. Our previous collagenase study showed that early HYPO aggravated bleeding[15], which did not occur presently in the whole blood model, but which may occur in patients experiencing re-bleeding[28] or in patients undergoing hemorrhagic transformation after ischemia. Safer treatment may be achieved through using alternate cooling methods (e.g., local cooling) as well as drug co-treatment (e.g., rFVIIa to treat coagulopathy). Interestingly, rFVIIa's activity is only slightly affected by cooling to 33°C[29], and it reduces bleeding in NORMO and HYPO pigs with liver injury[30]. Thus, rFVIIa and HYPO may be an especially effective approach for treating ICH.

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Table 4-1: Physiological variables measured before (top) and after ICH (bottom). Except for MABP (averaged throughout surgery), which was slightly higher in the HYPO-4 group, the values were within normal ranges and similar among groups both before and after ICH.

	NORMO	HYPO-1	HYPO-4
pH	7.42 ± 0.01	7.43 ± 0.01	7.42 ± 0.01
	7.40 ± 0.01	7.42 ± 0.03	7.42 ± 0.03
pCO_2 (mmHg)	42.9 ± 1.1	40.7 ± 0.7	42.9 ± 0.9
	46.7 ± 1.5	43.4 ± 0.8	44.4 ± 0.9
pO_2 (mmHg)	132.4 ± 2.3	132.1 ± 2.6	128.4 ± 2.6
	129.6 ± 2.2	130.9 ± 2.5	128.7 ± 3.1
Hb (g/dL)	13.7 ± 0.1	13.5 ± 0.5	14.1 ± 0.2
	13.3 ± 0.2	13.5 ± 0.6	13.8 ± 0.1
Glucose (mmol/L)	14.6 ± 0.4	13.5 ± 0.5	14.3 ± 0.3
	14.1 ± 0.5	13.6 ± 0.6	14.5 ± 0.4
MABP (mmHg)	96.7 ± 1.2	97.0 ± 1.4	102.2 ± 0.9

Figure 4-1: Core temperature (°C) for 4 days after ICH (Experiment 5; n = 21/group). Temperature profiles were similar in other experiments.

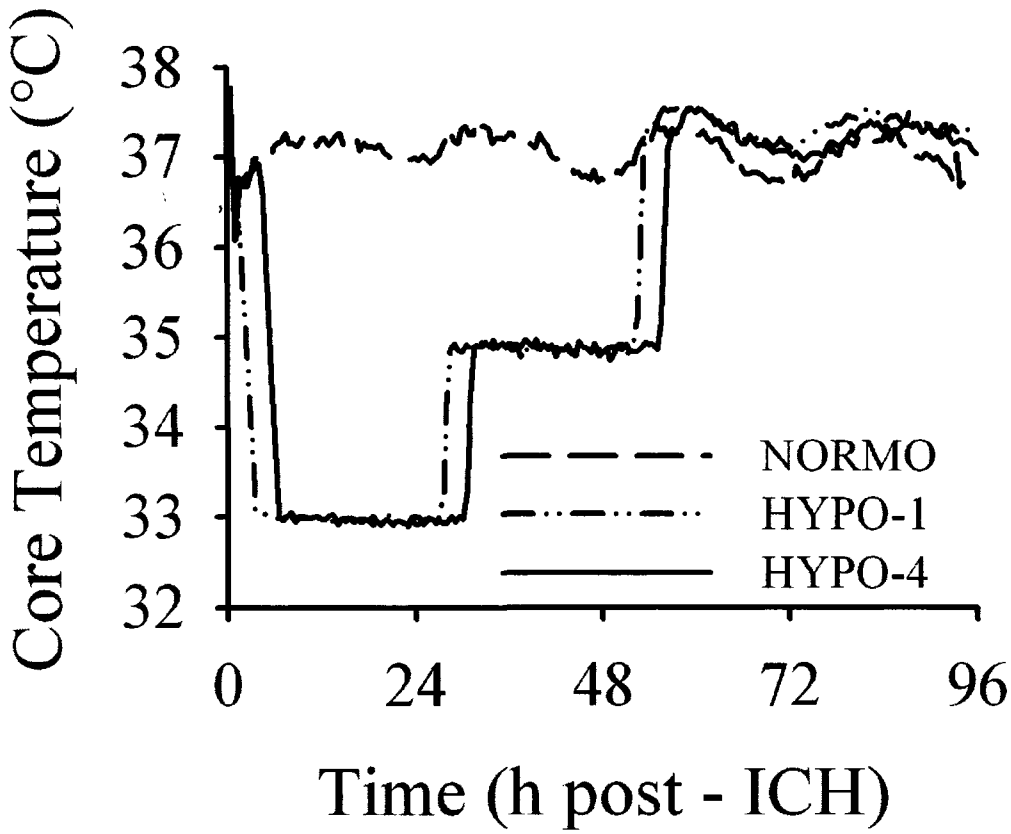


Figure 4-2: Evan's blue extravasation (μg dye/g tissue ± SEM) in the ipsilateral and contralateral hemispheres 48 hours after ICH (Experiment 2; n = 20/group). Ipsilateral extravasation was significantly reduced by HYPO–1 and HYPO–4 treatments.

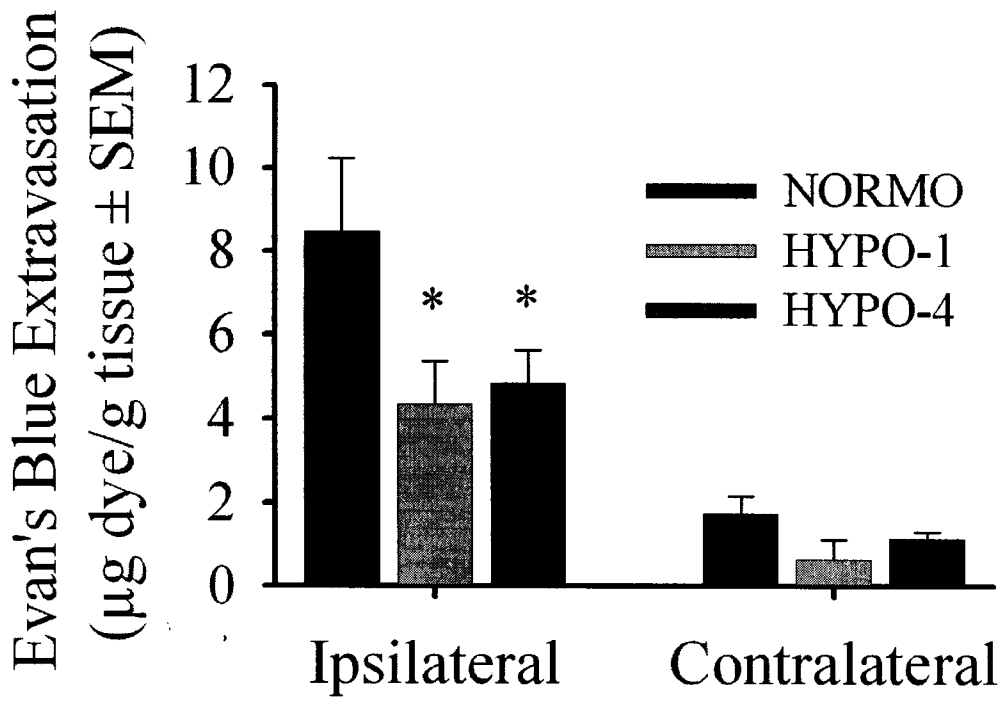


Figure 4-3: Number of neutrophils (A), iron-positive cells (B), and degenerating neurons (C) in the lesioned hemisphere 4 days post-ICH (mean ± SEM; Experiment 4; n = 10/group). Neutrophils were detected in the hematoma and surrounding tissue, whereas iron-positive cells surrounded the hematoma. Infiltration of neutrophils and macrophages was significantly reduced in the HYPO–1 and HYPO–4 groups. The number of degenerating neurons did not differ among groups.

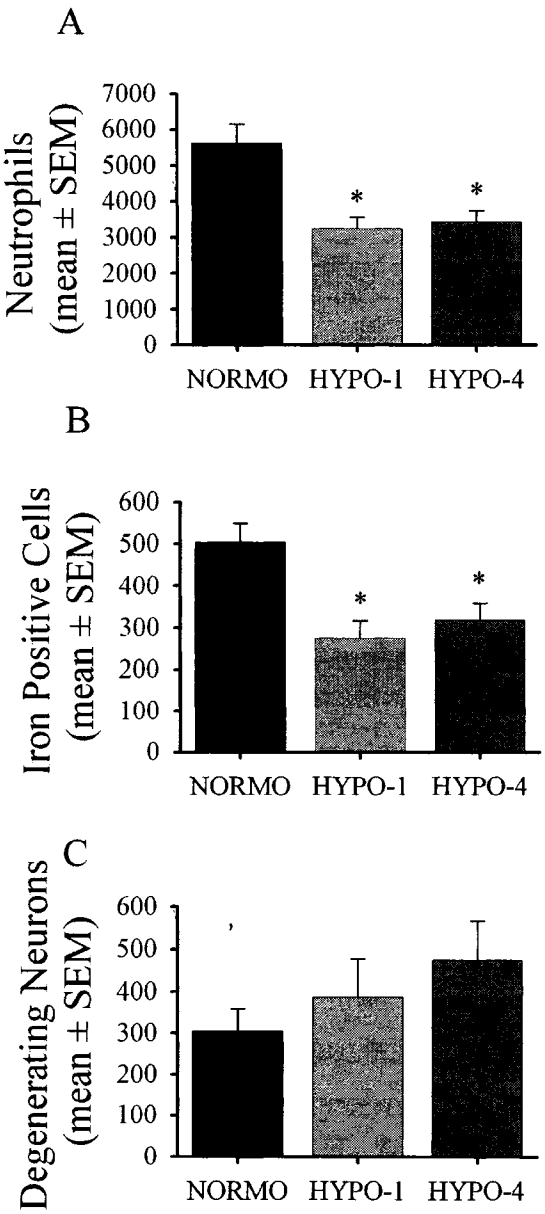


Figure 4-4: Hypothermia partially improves functional outcome in the staircase and ladder tests (% baseline ± SEM; Experiment 5; n = 21/group). The HYPO-1 group retrieved significantly more pellets on the first day of staircase testing (24 days post-ICH), but all groups showed good recovery thereafter (A). The HYPO-1 group made more successful steps with the contralateral hind limb than the other groups on day 7 (B). Limb-use asymmetry scores did not differ in the cylinder test (C).

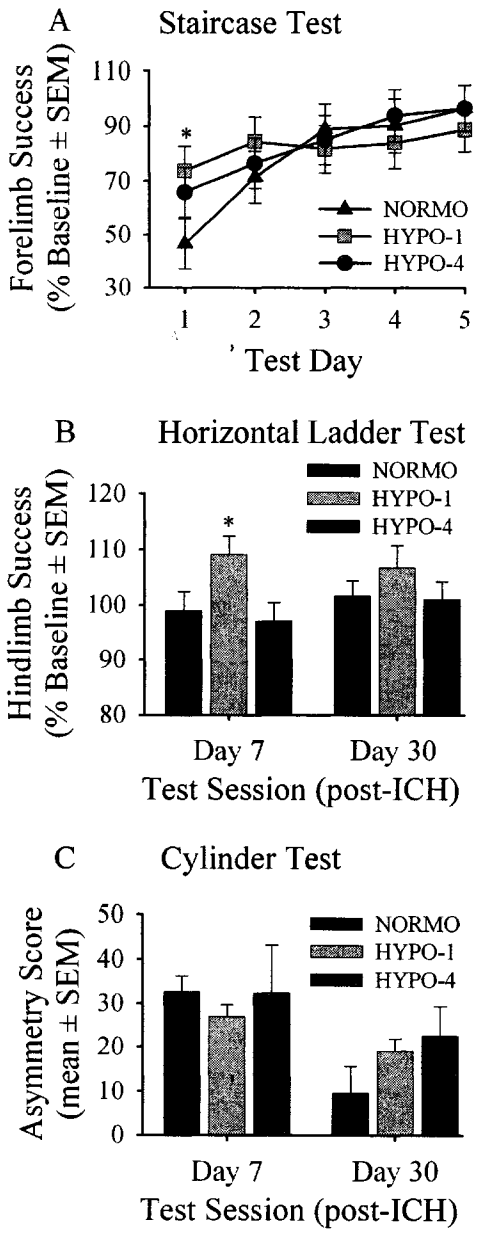
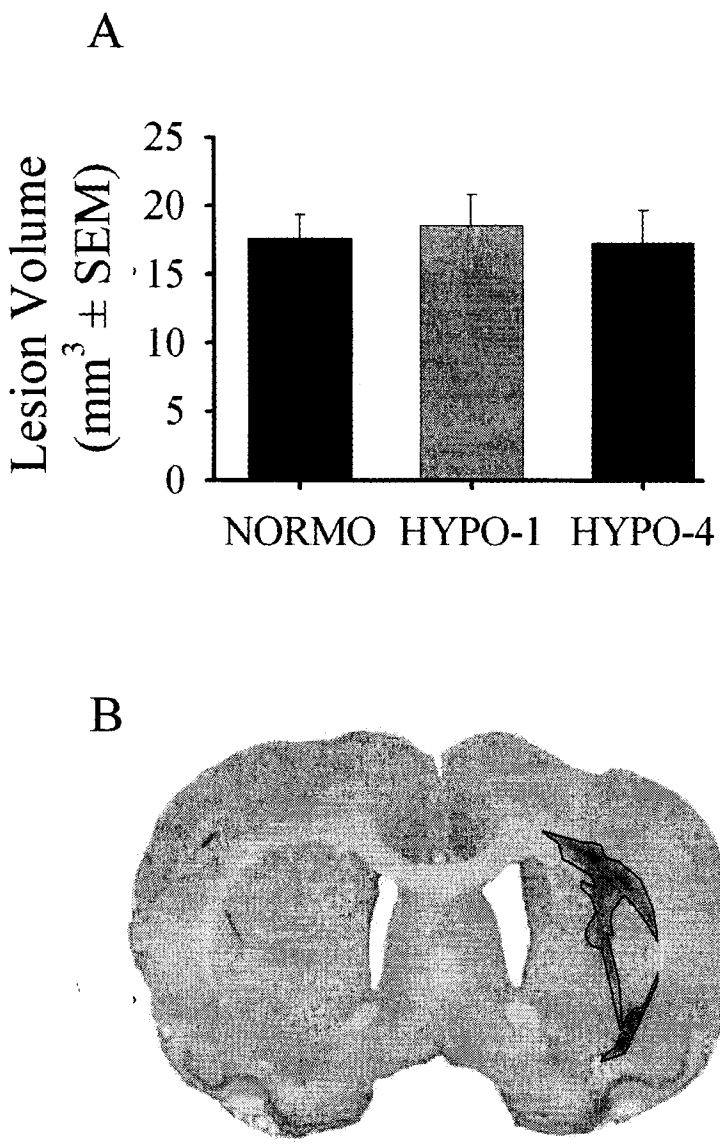


Figure 4-5: Hypothermia did not affect the volume of tissue lost (mm^3 ± SEM) 30 days after ICH (A; Experiment 5; n = 21/group). Lesions (B) included degenerating tissue (e.g., neurons, erythrocytes) and atrophy (e.g., ventriculomegaly).



Chapter 5: Mild to moderate hyperthermia does not worsen outcome following severe intracerebral hemorrhage in rats

A version of this chapter has been published. MacLellan, C.L. and Colbourne, F. 2005 *Journal of Cerebral Blood Flow and Metabolism*, 25: 1020-1029.

5-1 Introduction

Approximately 15 % of strokes in Western populations are the result of an intracerebral hemorrhage (ICH), which is the most devastating and least treatable type of stroke[1]. Current treatment of ICH is primarily supportive in nature (e.g., control of blood pressure), and outcome is generally poor. Despite the fact that increased body temperature frequently occurs in patients with an ICH[2], few studies have examined the prognostic significance of hyperthermia on ICH outcome. Several clinical studies of ICH patients with elevated body temperature found that hyperthermia does not independently predict mortality[2-5]. The small number of investigations and conflicting results necessitate further investigation to determine the significance of hyperthermia after ICH.

Contrary to discordant findings in ICH, the relationship between fever and worsened outcome in ischemic stroke patients appears clear. Elevated body temperature upon admission[4, 6] or in the days following ischemia[7, 8] increases morbidity and mortality. Given that hyperthermia of as little as 0.5°C significantly worsens outcome[6, 9], it is recommended that body temperature be maintained at normothermic levels after stroke[10]. However, it should be noted that there is no clinical evidence proving that anti-pyretic treatments improve outcome.

Likewise, small temperature elevations in rodent models of ischemic injury critically affect outcome (for review see[10]). Hyperthermia during or after global ischemia[11-14] exacerbates neuronal injury. Similarly, spontaneous[15-17] and externally induced hyperthermia[18, 19] increases focal ischemic injury, even when delayed for 24 h after ischemia[20]. Hyperthermia is thought to aggravate ischemic injury through several

mechanisms including: increased blood-brain barrier disruption[13, 21], enhanced release of excitotoxic neurotransmitters such as glutamate[22], increased production of free-radicals[23], increased spreading depression[24, 25], greater cytoskeletal degradation[26] and impaired metabolism[27].

In experimental ICH, the majority of damage is rapid and is caused by compression and displacement of brain tissue following the sudden development of an intracerebral mass. However, several components of hemorrhage resemble ischemic injury. For instance, hemorrhage triggers secondary events such as disruption of the blood-brain barrier[28], edema formation[29], excessive production of free radicals[30], and pronounced inflammation[31, 32]. Accordingly, we examined whether mild to moderate hyperthermia affects factors known to influence outcome after ICH, such as inflammation[33] and bleeding[34, 35]. We assessed outcome at multiple time points using histopathology (e.g., lesion volume) and a battery of behavioural tests sensitive to hemorrhagic injury[33-36]. We hypothesized that hyperthermia would worsen functional and histological outcome in part by increasing inflammation.

5-2 Materials and Methods

5-2a Animals

All procedures followed the Canadian Council on Animal Care guidelines and were approved by a local animal care committee. A total of 144 male Sprague-Dawley rats were obtained locally and weighed between 350 – 400 g (~ 16 weeks old) at the time of ICH. Rats were randomly assigned to treatment groups.

There were four separate experiments in this study (detailed methods follow) that each tested up to four groups: normothermia (NORMO; temperature kept >36.5°C via 175 W infrared lamp), mild hyperthermia (38.5°C) for the first 24 h (HYP-1) or second 24 h period (HYP-2) following ICH and moderate brief hyperthermia (40°C for 3 h) starting 12 h post-ICH (HYP-3). Rats were otherwise maintained at normothermia for up to 48 h following ICH (e.g., for the first 24 h period in the HYP-2 treatment condition). In a preliminary study (N = 3 total), we examined the relationship between brain and body temperature under post-ICH hyperthermia conditions. Experiment 1 (N = 62) examined whether the three hyperthermia treatments worsen long-term (i.e., 30 d) histological and functional outcome after ICH (n = 15 – 16 each). We assessed skilled reaching, walking, swimming, limb use asymmetry, as well as the volume of tissue lost. In order to determine if there were transient effects of hyperthermia that could not be detected at later times, we assessed functional (walking and limb use asymmetry) and histological (lesion volume) outcome 7 days following ICH in NORMO (n = 8) and HYP-1 (n = 8) treatment conditions (Experiment 2). In Experiment 3, we measured neurological deficits 1 – 4 days after ICH and assessed parenchymal infiltration of macrophages and neutrophils at 2 and 4 days in NORMO (n = 12), HYP-1 (n = 12) and

HYP-3 (n = 13) rats. In Experiment 4 (NORMO, n = 8; HYP-1, n = 9; HYP-3, n = 9) we used a spectrophotometric assay of hemoglobin[34] to determine whether hyperthermia affects the extravasation of blood into the brain after ICH.

5-2b Surgical Procedures

All surgical procedures were performed under isoflurane anesthesia (4 % induction; 2 % maintenance in 70 % N_2O and 30 % O_2) using aseptic technique.

Telemetry Temperature Probe Implantation

All animals had core temperature telemetry probes (model TA10TA-F40; Transoma Medical, St. Paul, MN, USA) implanted into the peritoneal cavity three days before ICH surgery as described previously[34]. After surgery, rats were individually housed on receivers interfaced to a computer running A.R.T. software (Transoma Medical) that sampled temperature every 30 seconds. Baseline core temperature was recorded for a complete 24 h period prior to ICH. Following ICH, temperature was monitored for up to seven days (Experiments 1 and 2) or until euthanasia in experiments with shorter survival times. In Experiment 1, core temperature telemetry probes were removed under brief anesthesia seven days following ICH.

In a pilot study, several rats were implanted with a core temperature telemetry probe as well as a telemetry brain probe (model XM-FH; Mini Mitter CO., Inc., Bend, OR, USA) immediately following ICH so that the temperature of the contralateral dorsal striatum was also measured in NORMO, HYP-1 and HYP-3 rats (n = 1 each). Continuous and simultaneous sampling of brain and core temperature is not possible owing to signal interference. Instead, core temperature was sampled every 30 sec followed by intermittent (every 4 h) brain temperature recordings[37]. We found a strong

relationship between brain and core temperature measured near-simultaneously after ICH (NORMO; r = 0.892), though brain temperature was ~ 0.9 °C lower than core temperature (mean of 36.5 vs. 37.4 °C, respectively). However, as core temperature was elevated in the HYP-1 and HYP-3 rats, brain temperature also increased and eventually exceeded core temperature. Due to technical difficulty in securing a probe on the head while allowing access to the striatum for collagenase infusion, we elected to regulate core temperature in other experiments.

Intracerebral Hemorrhage

All rats were anesthetized and placed in a stereotaxic frame. A hole was drilled 3 mm lateral (right) to Bregma and a 26 - gauge needle (Hamilton syringe, Hamilton, Reno, NV, USA) was lowered 5.5 mm below the surface of the skull. To create an ICH, 0.8 μL of sterile saline containing 0.16 U bacterial collagenase (Type IV-S, Sigma, Oakville, ONT, Canada) was infused into the striatum over 5 min. Following infusion, the needle remained in place for an additional 5 min, then a metal screw (model MX-080-2, Small Parts, Miami Lakes, FL, USA) was used to seal the hole. The scalp wound was treated with Marcaine (Sanofi, Markham, ONT, Canada) and closed with staples. Rats were not intubated during surgery in order to minimize invasiveness and duration of anesthetic.

5-2c Behavioural Testing

Staircase Task (Experiment 1)

Three weeks prior to ICH surgery (Experiment 1), rats were food deprived to 90% of free-feeding weight and trained in the staircase test, to measure independent forelimb reaching ability. Briefly, rats received two 15 min trials per day separated by 4 – 5 h, 5 days per week[34, 36]. Ten rats failed to reach the criterion of 9 pellets (45 mg each; Bio-

Serv, Frenchtown, NJ, USA) per side out of a possible 21 by the last three consecutive days of training, and were thus excluded from just this test.

Skilled reaching deficits were assessed 24 – 28 days post-ICH. Testing consisted of 10 trials (two 15 min trials per day, separated by 4 – 5 h) during which rats were maintained at 90% of their free-feeding weight. We recorded the total number of pellets retrieved on each side for each trial, and expressed performance as a percent of baseline (average of the last 10 training trials).

Swimming Task (Experiment 1)

Rats in Experiment 1 were trained to swim to a visible platform located at one end of a rectangular aquarium (length: 123, width: 46, height: 57 cm) for three days prior to core probe implantation. Each rat was trained (~ 10 consecutive trials / day for 2 days) to swim from one end of the tank directly to the platform without touching the walls[38]. Three baseline trials were videotaped for each animal on the third day. Normally, the hind limbs are used to propel the rat through the water while the forelimbs are held under the jaw. Damage to the motor cortex, often accompanied by injury to the dorsolateral striatum, causes asymmetry in forelimb inhibition and use of the impaired limb during swimming[38]. Thirty days after ICH, three trials were videotaped for each animal as they swam directly to the platform. We recorded the number of strokes made with each forelimb and calculated an asymmetry score for forelimb inhibition:

Number of contralateral forelimb strokes – number of ipsilateral forelimb strokes

Forelimb Use Asymmetry Test (Experiments 1 and 2)

Seven (Experiment 2) or 30 days (Experiment 1) after ICH, rats individually explored a transparent cylinder (45 cm in height and 20 cm in diameter) for 10 min. A video camera set up below the cylinder recorded each animal's movements, which were analyzed according to our previous work[34]. Briefly, independent forelimb use for weight support during exploration of the walls was expressed as:

(Number of contacts with contralateral limb / ipsilateral + contralateral limb use) × 100

Horizontal Ladder Walking Test (Experiments 1 and 2)

At 7 (Experiment 2) or 30 days (Experiment 1) after ICH, rats were videotaped walking across a 1 m horizontal ladder with variably spaced bars ranging from 3 to 5 cm apart. For each forelimb, the percentage of footfalls (limb slips completely through the bars) made over four trials was determined[34, 36].

Neurological Deficits Score (NDS; Experiment 3)

Neurological deficits were measured 1 – 4 days after ICH[32, 33] in Experiment 3. Tests included (1) spontaneous ipsilateral circling, graded from 0 for no circling to 3 for continuous circling; (2) hind limb retraction, graded from 0 for immediate replacement to 3 for no retraction after the hind limb was moved laterally 2 - 3 cm; (3) bilateral forepaw grasp, graded from 0 for normal grasping behaviour to 3 for a rat unable to grasp with the forelimbs; (4) contralateral forelimb flexion, graded from 0 for uniform extension of forelimbs to 2 for full wrist flexion and shoulder adduction when the rat was gently lifted by the base of the tail; and (5) ability to walk a 70 cm long × 2.4 cm wide beam; graded

from 0 for a rat that readily traversed the beam to 3 for a rat unable to stay on the beam for > 10 s. Scores for each component were added for a maximum of 14 (greatest impairment).

5-2d Histopathology

Volume of Brain Tissue Destruction (Experiment 1 and 2)

Rats were euthanized 7 (Experiment 2) or 30 days (Experiment 1) after ICH with sodium pentobarbital (80 mg / kg) and were transcardially perfused with saline and then 10 % neutral buffered formalin. Forty-μm coronal sections were taken with a cryostat every 600 μm and stained with cresyl violet. Using Scion Image J 4.0 (Scion Corporation, Frederick, MD, USA) the volume of lesion plus atrophy (e.g. ventricular enlargement) was quantified and expressed as follows[34]:

Volume of tissue lost = remaining volume of normal hemisphere – remaining volume of lesioned hemisphere.

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 analyzed.

Inflammatory Cell Infiltration (Experiment 3)

Rats in Experiment 3 were euthanized as described above 2 or 4 days following ICH. A 4 mm thick section of tissue encompassing the lesion was taken from each brain and embedded in paraffin. Ten μm sections were taken with a microtome and stained with Perl's Prussian blue for ferric iron and Leder's stain[39] for chloracetate esterase

activity (Kit 91-C, Sigma, Oakville, ONT, Canada). These particular stains have been used to quantify components of inflammation after ICH in several other studies[32, 33]. In consecutive sections, we counted the total number of activated macrophages (stained blue) and viable neutrophils (stained red) in the damaged hemisphere at the level of maximum hematoma diameter.

Spectrophotometric Assay of Hemoglobin (Experiment 4)

Rats in Experiment 4 were subjected to an ICH and then 24 h later were overdosed with sodium pentobarbital and transcardially perfused with 100 mL of 0.9 % saline. Brains were extracted, dissected free of olfactory bulbs and cerebellum, and homogenized (Bio-Spec, Racine, WI, USA) for 60 seconds in a test tube containing distilled water (total volume of 3 mL). After centrifugation (15,800 × g for 30 minutes; model CR20B2, Hitachi, Japan), Drabkin's reagent (400 μL; Sigma) was added to 100 μL aliquots of the supernatant (at least four samples per brain) and allowed to react for 15 minutes. The absorbance of this solution was read using a spectrophotometer (540 nm; model DU-8; Beckman Coulter Ltd., London, U.K.) and the amount of blood in each brain was calculated using a curve generated previously using known blood volumes[34].

5-2e Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Data were analyzed using a one-way ANOVA or with t-tests (two-tailed) that did not assume equality of variance in the case of a significant Levene's test (SPSS, v. 11.5). The Kruskal – Wallis test was used for heterogeneous data (e.g., ipsilateral limb error rate in the ladder task) and non-parametric data (e.g., NDS).

5-3 Results

5-3a Experiment 1

Baseline core temperature recorded before surgery was similar among groups (overall mean = 37.0 ± 0.02 °C; data not shown), and was regulated as desired after ICH (Fig 1a). On the first day after ICH, the NORMO group was slightly (0.4 °C) but significantly warmer than baseline temperature ($P < 0.001$; Fig 1b). Temperature was forcibly elevated (vs. baseline) in the HYP-1, HYP-2 and HYP-3 groups by 1.4 °C, 1.4 °C, and 2.9 °C, respectively (average of hyperthermia period). One HYP-3 rat died one day after ICH of unknown cause.

Rats performed similarly during staircase training (data not shown). Intracerebral hemorrhage (NORMO) caused significant reaching impairments with the contralateral (31.8 % of baseline; $P < 0.001$ vs. baseline) and ipsilateral (78.5 % of baseline; $P = 0.019$) forelimbs during testing (days 24 - 28 post - ICH). The hyperthermia treatments did not affect reaching success with either forelimb ($P \geq 0.151$; Fig 2). Untreated ICH caused significant impairments with the contralateral limbs ($P < 0.001$ vs. ipsilateral limb) in the ladder-walking test. NORMO rats had a mean slip rate of 19.6 ± 2.7 % with the contralateral forelimb (Fig 3a). Performance was similar (mean of 18.6 ± 3.0 %) with the contralateral hind limb. Error rates were small with the ipsilateral forelimb (overall mean = 0.8 ± 0.2 %) and hind limb (overall mean = 1.7 ± 0.6 %). The hyperthermia treatments did not significantly affect error rate of any limb ($P \geq 0.156$). Similarly, there was no main effect of group in the limb-use asymmetry task ($P = 0.114$; Fig 4a). After ICH, rats displayed a substantial asymmetry favouring use of the ipsilateral limb (overall mean = 89.2 ± 2.1 %). Finally, all groups exhibited an asymmetry in forepaw inhibition

in the swim test after ICH (overall mean = 4.8 ± 0.7 strokes; $P \leq 0.018$ vs. baseline performance), however there was no difference among groups ($P = 0.293$).

ICH resulted in damage primarily to the striatum, but also to the internal capsule, corpus callosum, globus pallidus, and thalamus. Tissue lost 30 days after ICH was not significantly different among groups (overall mean = 73.3 ± 2.8 mm^3; $P = 0.356$; Fig 5a and b).

5-3b Experiment 2

Temperature of NORMO and HYP-1 groups was regulated according to Experiment 1 (data not shown). Seven days after ICH, NORMO rats had a contralateral forelimb error rate of 17.0 ± 2.2 % in the ladder test (Fig 3b). Performance was similar (20.9 ± 7.0 %) with the contralateral forelimb in the HYP–1 group. Error rates of each limb were similar between groups ($P \geq 0.285$). One HYP–1 rat was excluded from this task for failure to cross the ladder. Both the NORMO (90.7 ± 7.3 %) and HYP-1 (95.3 ± 2.1 %) groups displayed increased usage of their ipsilateral forelimb in the limb-use asymmetry task, but were not significantly different from each other ($P = 0.493$; Fig 4b). One HYP–1 and two NORMO rats did not explore the cylinder wall and were thus excluded from analysis. The NORMO group sustained a 62.6 ± 7.5 mm^3 lesion by 7 days post-ICH (Fig 5c and d). The HYP-1 treatment did not increase the volume of tissue lost (59.5 ± 6.0 mm^3; $P = 0.752$).

5-3c Experiment 3

Temperature was regulated as described above (data not shown). Rats exhibited significant neurological deficits 1 - 4 days after ICH ($P < 0.001$ vs. baseline; data not shown). The HYP-1 and HYP-3 conditions did not affect neurological deficit scores on

any day ($P > 0.253$; Fig 6). The number of macrophages observed in the injured hemisphere was not significantly different among groups at either two ($P = 0.798$) or four days ($P = 0.489$) following ICH (Fig 7). Similarly, hyperthermia did not significantly affect the number of neutrophils present at two (overall mean 951.0 ± 130.2 neutrophils; $P = 0.845$) or four days (overall mean 1095.6 ± 271.7; $P = 0.573$). Four brains could not be evaluated due to poor tissue quality.

5-3d Experiment 4

Post-ICH temperature was regulated as in Experiment 1 (data not shown). The NORMO group had 56.5 ± 12.0 μL of blood in the brain 24 h after ICH. This was not significantly different ($P = 0.587$) in the HYP-1 (39.2 ± 6.2 μL) or HYP-3 groups (42.9 ± 15.9 μL). One HYP-3 rat died in this experiment and was not included in the analysis.

5-4 Discussion

This is the first study to assess the effects of mild to moderate hyperthermia on outcome following experimental ICH. Contrary to our expectations, hyperthermia did not aggravate bleeding, macrophage or neutrophil infiltration, and had no discernable effect on long-term histological or functional outcome. These findings contrast with the overwhelming evidence that even mild elevations in temperature markedly worsen outcome after cerebral ischemia in humans and rodents. However, our findings are consistent with several clinical studies of ICH in which hyperthermia did not independently predict mortality[3-5].

The differing effects of hyperthermia after ischemic and hemorrhagic stroke are likely due to fundamental differences in the underlying pathophysiology. For instance, it is the penumbra (mildly ischemic region) that is recruited by hyperthermia after focal

ischemia[17]. Notably, experimental[40] and clinical ICH studies[41] fail to find a significant penumbral region surrounding the hematoma. As well, the majority of hemorrhagic injury occurs rapidly and is caused by direct tissue destruction and space-occupying effects of the hematoma. Thus, mechanisms thought to aggravate ischemic injury (e.g., increased inflammation) may not *substantially* worsen outcome after ICH. Indeed, many anti-inflammatory agents fail to lesson lesion size after ICH[33, 42]. Additionally, hyperthermia aggravates ischemic injury through increased excitotoxicity[10]; yet the role of the less dramatic increases in excitatory amino acids found soon after ICH has yet to be defined[43]. Accordingly, we expect that mild and moderate hyperthermia, under the conditions tested, do not substantially and directly aggravate key mechanisms contributing to injury after ICH (e.g., bleeding, mechanical destruction). Furthermore, as the insult was not worsened (i.e., volume of injury did not change), one would not expect a concomitant increase in inflammation. Perhaps mild hyperthermia does not directly aggravate inflammation after focal cerebral ischemia. Instead, an enlarged lesion (e.g. due to enhanced excitotoxicity) may result in greater inflammation, which may then act to further worsen outcome.

Hyperthermia may aggravate mild hemorrhagic insults, whereas more severe insults may not be noticeably altered. Although the insult produced in this study is large, we have previously created larger lesions (e.g., greater rostral – caudal extension of damage) with more severe impairments (e.g., in skilled reaching) and greater mortality (unpublished data). Thus, injury and mortality could have been increased in this study. Hyperthermia may have also affected other mechanisms that we did not assess, or perhaps there was an effect that would have been detected with other functional tests,

histological measures, or additional survival times. We evaluated functional outcome using five tests sensitive to hemorrhagic injury[33, 34, 36] and measured the volume of tissue lost at a short (e.g., 7 d) and long - term (e.g., 30 d) survival. Furthermore, we assessed the effects of hyperthermia on mechanisms such as bleeding and inflammation, which contribute to hemorrhagic injury. The clear lack of effect suggests that mild to moderate hyperthermia does not worsen histological or functional outcome, and other mechanisms or undetected effects likely do not contribute significantly to injury.

Near-simultaneous measurement of core and brain temperature after ICH in a pilot study showed that brain temperature was slightly cooler (by < 1 °C) than body temperature, as previously shown using this method in normal rats[37]. In accordance with previous work from this[44] and other labs[20], we found that increasing body temperature disrupts the normally good relationship between body and brain temperature. Indeed, brain temperature was relatively overheated (vs. core temperature) when body temperature was elevated via infrared lamps. Further study is needed to determine the relationship between body and brain temperature when other methods of temperature measurement (e.g., rectal or temporalis muscle) and regulation (e.g., using a heating pad) are employed. For example, perhaps an even greater overheating of the brain occurred in ischemic studies that used rectal temperature measurements or alternative heating methods.

Our previous work demonstrated that delayed hypothermia reduces the volume of tissue lost and improves functional outcome in this model of ICH[34]. However, hypothermia induced soon after ICH increases the volume of extravasated blood due to adverse physiological effects of the treatment (e.g., elevated blood pressure).

Additionally, we recently showed that 17-ß estradiol attenuates bleeding in this model and thereby lessens injury[35]. Accordingly, because injury and bleeding can be improved or worsened, it is reasonable to expect that hyperthermia *could* have affected outcome in this model of ICH. Differences in experimental design may explain why hypothermia affected outcome whereas hyperthermia did not. Notably, the magnitude of the enforced temperature change was not equivalent in these studies. Temperature was reduced by up to 4 °C (to 33 °C) during prolonged hypothermia[34] whereas temperature was persistently elevated by 1.5 °C in the HYP-1 and HYP-2 conditions in the present study. Second, the duration of temperature regulation was not the same in these studies. Thus, greater effects may have been detected had we kept rats at greater hyperthermic levels or for more protracted periods. It should be noted, however, that infarct volume and neurological deficits are increased when brain temperature is elevated to 40 °C for 3 h starting 24 h after focal ischemia in rats[20]. Our similar HYP-3 treatment was induced *sooner* after ICH, yet had no discernable effect on outcome. Finally, in our opinion, prolonged mild hypothermia provides substantially less benefit after ICH[34] than after global (e.g.,[45]) and focal (e.g.,[46]) cerebral ischemia. Hemorrhagic injury simply appears to be less sensitive to temperature manipulations, which is in agreement with the majority of clinical studies on hyperthermia after ICH.

Future studies should evaluate alternate causes (e.g., infection) and complications (e.g., cardiovascular instability) of hyperthermia as well as its effects on other hemorrhagic events such as hemorrhagic transformation after ischemic stroke and traumatic brain injury (e.g., see [47]). Furthermore, hyperthermia may differentially affect male and female rodents as gender[48] and estrogen affects ICH insults. Such experimental

studies are needed in conjunction with clinical findings to clarify the independent significance of hyperthermia on mortality and functional outcome after ICH.

In summary, mild to moderate increases in body temperature did not worsen brain injury, bleeding, inflammation, or functional deficits after ICH in rats. These results are consistent with clinical findings that hyperthermia is not an independent prognostic factor in ICH patients. The failure of hyperthermia to critically affect outcome in this study suggests that treating mild to moderate hyperthermia may provide no cytoprotective benefit. However, treating the causes of fever (e.g., infection) or life – threatening fever is recommended.

5-5 References

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Table 5-1: Summary of treatment conditions and endpoints in each experiment. Number in parentheses denotes group size.

Experiment	Treatment Condition	Endpoint	Survival
Preliminary Study	NORMO (1), HYP-1 (1), HYP- 3 (1)	Body vs. Brain Temperature	32 h
1	NORMO (16), HYP-1 (15), HYP-2 (15), HYP-3 (16)	Function (staircase, cylinder, ladder and swim) and Histology (lesion volume)	30 d
2	NORMO (8), HYP-1 (8)	Function (cylinder, ladder) and Histology (lesion volume)	7 d
3	NORMO (12), HYP-1 (12), HYP-3 (13)	Function (NDS) and Histology (macrophages and neutrophils)	2 or 4 d
4	NORMO (8), HYP-1 (9), HYP-3 (9)	Cerebral Blood Volume	1 d

Figure 5-1: Core temperature (°C) averaged every 30 min (sampled every 30 s) for 4 days after ICH (A). Temperature from days 5 to 7 days was similar (data not shown). The corrected magnitude of temperature elevation after ICH is shown in B (i.e., temperature after ICH – baseline, which controlled for time of day). See Methods for temperature manipulations.

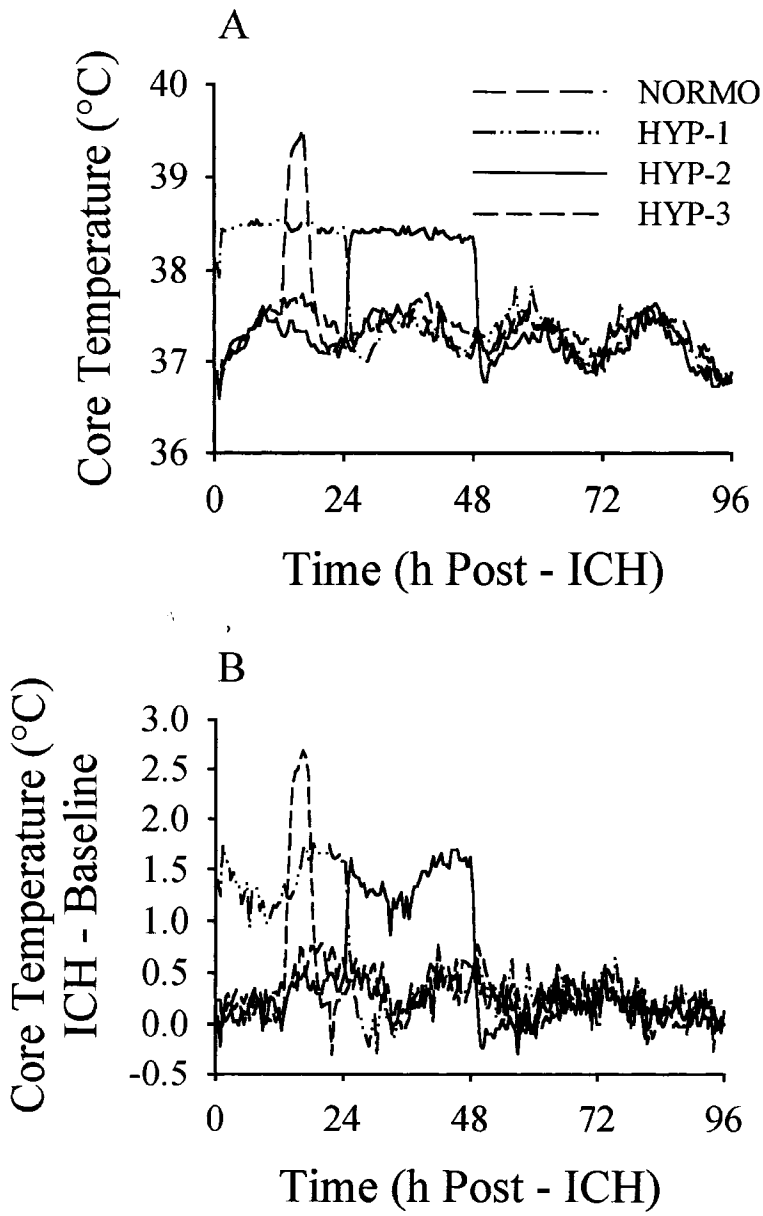


Figure 5-2: Reaching success (% baseline) with the ipsilateral (A) and contralateral (B) forelimbs in the staircase apparatus over 10 test trials 24 – 28 days after ICH (Experiment 1). There was no significant difference among groups with either forelimb.

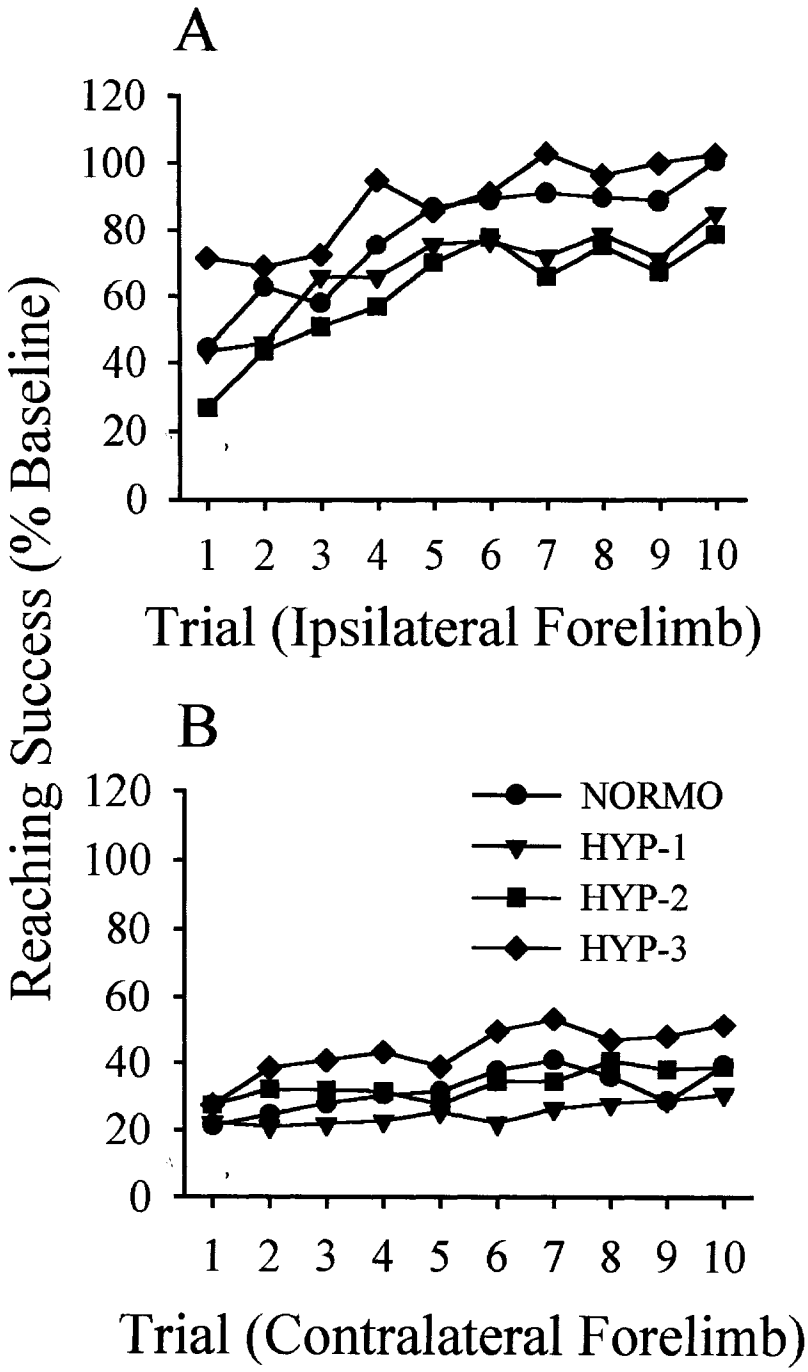


Figure 5-3: Error rate (% falls through bars ± SEM) made with the contralateral forelimb on the horizontal ladder 30 days (A; Experiment 1) or 7 days (B; Experiment 2) after ICH. Hyperthermia did not increase error rate of any limb at either time point.

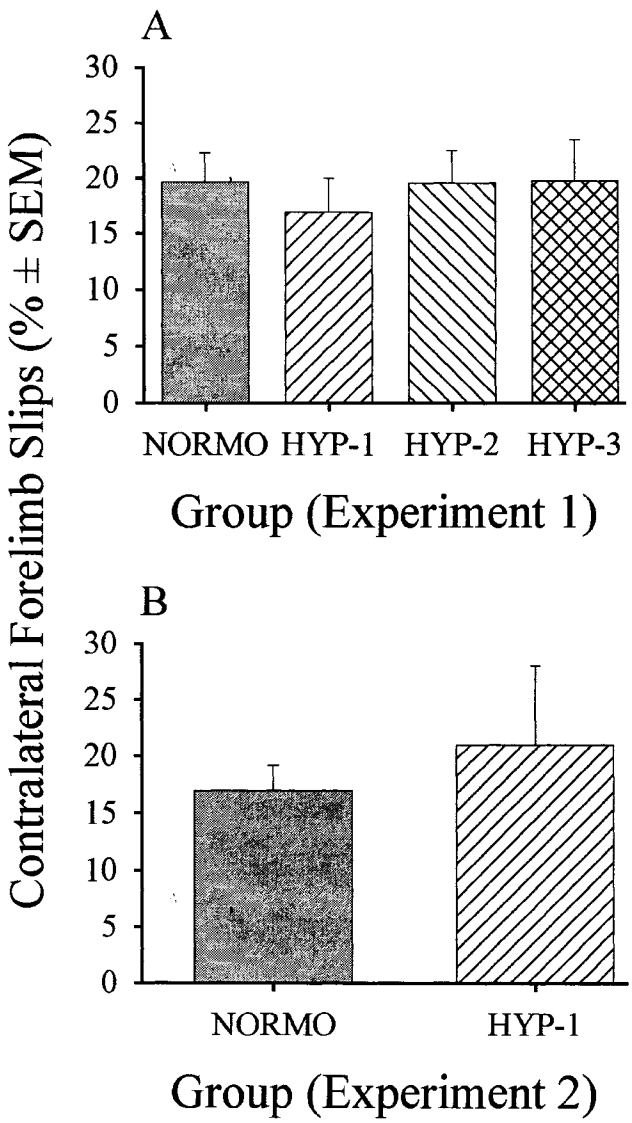


Figure 5-4: Spontaneous ipsilateral forelimb use (% independent limb use ± SEM) in the cylinder test 30 days (A; Experiment 1) and 7 days (B; Experiment 2) following ICH. There were no group differences at either time.

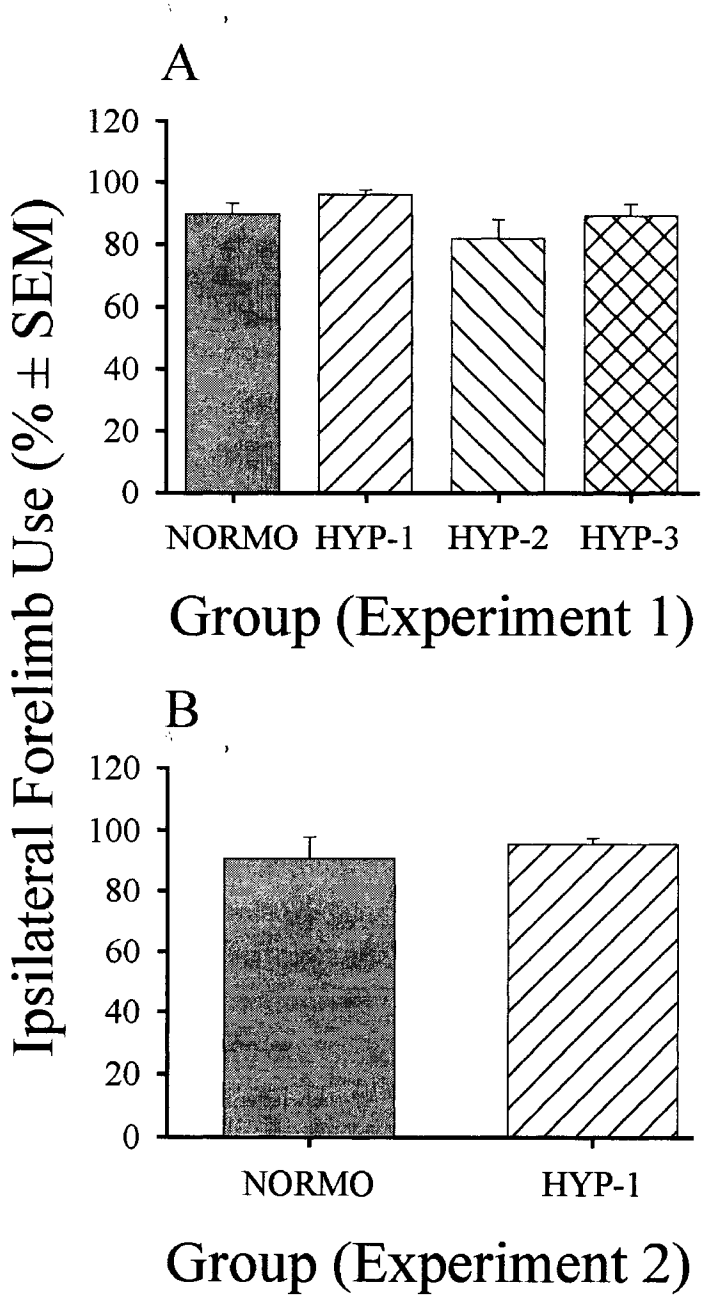


Figure 5-5: Volume (mm^3 ± SEM) of tissue lost 30 days (A; Experiment 1) and 7 days (C; Experiment 2) after ICH. Hyperthermia did not increase tissue loss at either time. Photomicrographs illustrate the average lesion area following ICH. The area of degenerating tissue (cellular debris including neurons, erythrocytes, inflammatory cells, etc.) observed 7 days (D) after ICH becomes a cavity by 30 days (B).

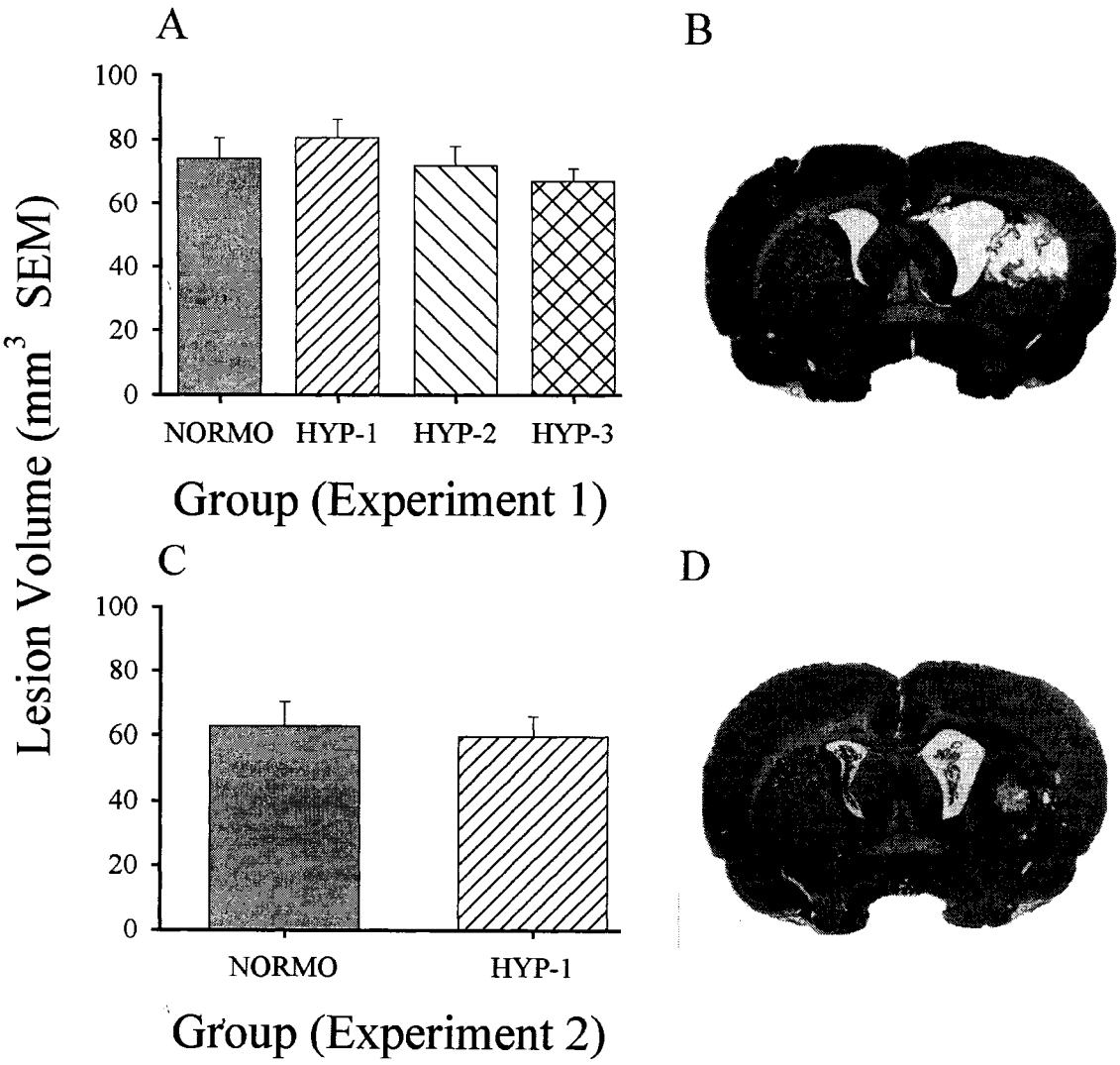


Figure 5-6: Neurological deficit scores (bar denotes group median) at 2 days following ICH (Experiment 3). Data were similar on other days (data not shown). Hyperthermia conditions did not affect neurological deficits at any time.

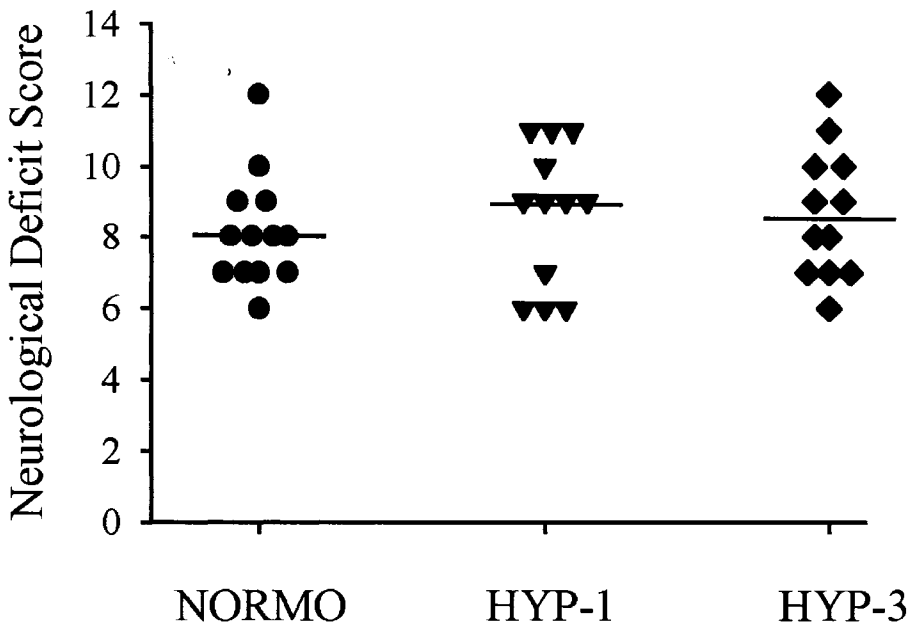
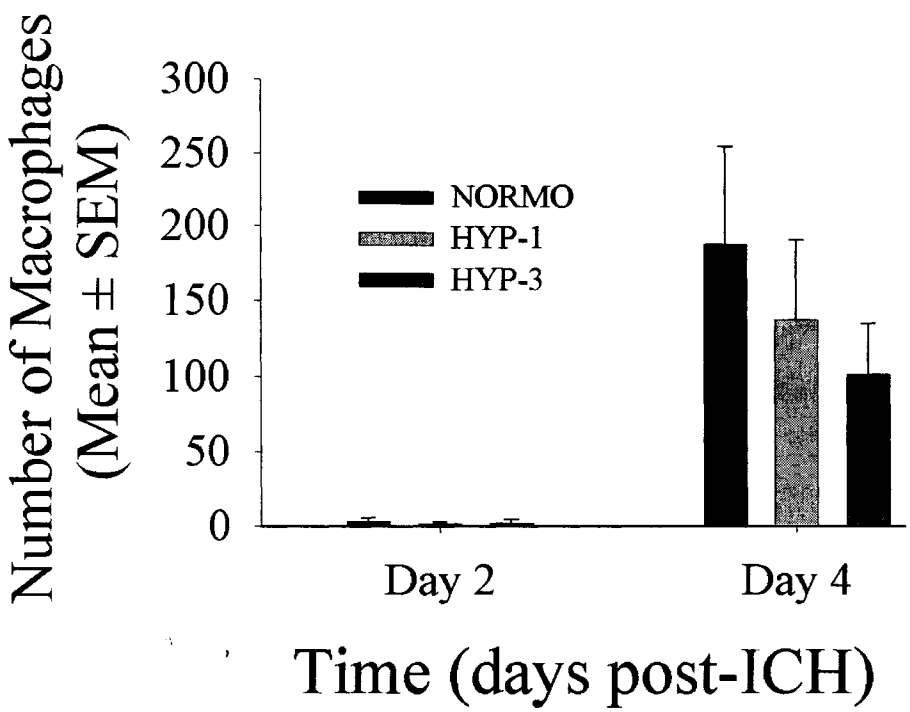


Figure 5-7: Number of activated macrophages (mean ± SEM; Experiment 3) in the lesioned hemisphere 2 and 4 days following ICH. There were no group differences at either time.



Chapter 6: Gauging recovery after hemorrhagic stroke in rats: Implications for cytoprotection studies.

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6-1 Introduction

Ischemic and hemorrhagic stroke are among the leading causes of death and disability. Accordingly, there is a great need for stroke models to assess putative cytoprotectants (neuroprotection). Unfortunately, despite much progress elucidating the pathophysiology of stroke, there has been little translation from experiments to patient treatment. These failures have been attributed to limitations with both basic and clinical studies[1-4]. For instance, long delays between stroke onset and treatment occurred in many clinical trials. Of the several major concerns raised with experimental studies[5], the oft failure to gauge functional therapeutic efficacy stands out[6-9]. Functional recovery is the more important clinical endpoint, yet investigators often quantify cell death as their sole index of treatment efficacy[6].

While a reduction in cell death will likely improve functional recovery, there are several reasons why this is not always true. For instance, tissue may remain viable, but function abnormally[10]. Furthermore, damage to regions distal to an infarct, such as axonal degeneration and atrophy, may go undetected, but contribute to impairment[7]. Likewise, spontaneous recovery and compensation further obfuscate the relationship between behavior and cell death. Thus, effective assessment of any therapy necessitates a rigorous examination of behavioral recovery in addition to histological measurements of injury because only those cytoprotectants that improve both in animal models are likely to improve outcome in humans.

Given the need for functional assessment, it is not surprising that many tests sensitive to functional deficits after ischemic stroke have been identified for rodents[11-13]. While most strokes are ischemic, approximately 15% are hemorrhagic and many

ischemic strokes undergo hemorrhagic transformation[14]. Accordingly, studies have identified tests that are sensitive to striatal intracerebral hemorrhage (ICH) in rodents[15, 16]. These studies however, have not thoroughly assessed whether such tests are widely appropriate for gauging variations in the volume of injury (e.g., cytoprotection). Functional tests must not only act as lesion detectors, they must also distinguish among gradations in injury and do so over time in order to track recovery.

This study examined whether a large battery of tests (e.g., skilled reaching, walking; see Methods), which are each sensitive to ischemic and traumatic damage to the motor system, could predict histological outcome after ICH in rats. We created a range of lesion sizes by varying the dose of collagenase used to produce the ICH, and then assessed performance over one month. The gradations in lesion size from large to medium to small represent sizeable and statistically significant reductions in damage that would presumably have great clinical benefit. In this regard, we aimed to evaluate whether marked differences (reductions) in brain injury, such as those afforded by cytoprotective agents, could be detected with a single test or combination of tests.

6-2 Materials and Methods

6-2a Animals

Sixty male Sprague-Dawley rats weighing approximately 250 g (~10 weeks old; obtained locally) were used in this study. All procedures were in accordance with the Canadian Council on Animal Care guidelines, and were approved by Biological Sciences Animal Policy and Welfare Committee at the University of Alberta.

6-2b Behavioural Training

Baseline performance for the horizontal ladder, forelimb use asymmetry (cylinder), adhesive tape removal, and beam walking tests was measured the day before surgery. Neurological deficits were also assessed. Other tests required more extensive training as detailed below.

Staircase Test

Rats were food deprived to 90% of their free-feeding weight over three days and then trained in the staircase test of independent forelimb reaching ability[17]. Food restriction took into account the natural gain in body weight during this period. For the test, each rat is placed into a plexiglass box (length: 30 cm, width: 6.8 cm, height: 12 cm) and rests on an elevated platform with 7 stairs descending on each side. Each stair has a food well baited with 3 food pellets (45 mg each, Bio-Serv, Frenchtown, NJ, U.S.A.). Pellets on the left stairs may only be retrieved with the left paw, and on the right stairs using the right paw. Recording the number of pellets retrieved with each forelimb assesses skilled reaching ability. Rats were trained twice daily for 5 days a week over 3 weeks, and were excluded from this test if they did not retrieve an average of 8 pellets per side or more out of a possible 21 on 3 consecutive days.

Single Pellet Test

Rats were maintained at 90% of free feeding weight and trained in the single pellet test 5 days per week over 3 weeks with 20 reaches per day. Briefly, rats were placed in a 14 cm × 60 cm box and trained to reach through a 1 cm wide opening to retrieve a food pellet (45 mg each, Bio-Serv, Frenchtown, NJ, U.S.A.) placed on the ledge in front of the opening[18]. A reach was considered a success if the rat grasped the

pellet, brought it inside the box using its paw, and placed the pellet into its mouth. A reach on which an animal advanced the paw through the slot but missed the pellet or knocked it off the ledge was considered a failure. Performance on the last day of training was videotaped for kinematic analysis of reaching movements. The first three successful reaches for each rat were each analyzed qualitatively[19]. Briefly, a reaching movement was rated on a scale of 11 movement components: (1) orient to pellet, (2) limb lift, (3) digits close, (4) aim, (5) advance, (6) digits open, (7) pronation, (8) grasp, (9) supination I, (10) supination II, and (11) release. These movement components were further broken down into a total of 35 subcategories, which were graded 0 (loss of normal movement), 0.5 (impaired movement pattern), or 1 (normal movement). A score of 35 indicated a perfect reach.

Forelimb Inhibition Test (Swimming)

Rats were trained over 2 days (10 trials / day) to swim to a visible, above-water platform located at one end of an aquarium (length: 123, width: 46, height: 57 cm). Three baseline trials were videotaped on the third day. Rats normally inhibit the forelimbs as they swim, propelling themselves with only their hind limbs[20]. A striatal ICH causes asymmetry in forelimb inhibition and use (positive sign) of the impaired forelimb during swimming[21]. A rat was excluded from analysis if after repeated trials it failed to swim directly to the platform without paddling along the walls of the tank (e.g., escape or circling behavior). The number of strokes made with each forelimb was recorded over 3 trials and an asymmetry score for forelimb inhibition was calculated as:

Σ (Number of contralateral forelimb strokes – number of ipsilateral forelimb strokes) / number of trials.

6-2c Surgery and Experimental Groups

Rats were anesthetized with isoflurane (induction 4 %, maintenance 1.5 – 2 % in 70 % N_2O and 30 % O_2) and placed in a stereotaxic frame. Body temperature was maintained near normothermia (37 °C) throughout surgery using a rectal probe and heating blanket. Under aseptic conditions, a midline scalp incision was made and a small hole drilled at 3.0 mm lateral to Bregma in the hemisphere contralateral to the preferred paw as determined during the single pellet task training. We created a range of insult severities using the well-characterized bacterial collagenase model of ICH[22-25]. Briefly, a 28-gauge Hamilton needle (Hamilton, Reno, NV, U.S.A.) was lowered 6.0 mm ventral to the surface of the skull and 1.0 μL of sterile saline containing 0.06 (MILD; n = 15), 0.12 (MODERATE; n = 15) or 0.18 U (SEVERE; n = 15) of bacterial collagenase (Type IV-S, Sigma Chemical Co, Oakville, Ontario, Canada) was infused into the striatum. A control group (SHAM; n = 15) received saline infusion only. A metal screw (Model MX-080-2; small parts, Miami Lakes, FL, U.S.A.) sealed the hole and Marcaine (Sanofi Canada, Markham, Ontario, Canada) was infiltrated into the area. The wound was closed with staples, and treated with antibiotic ointment. Anesthesia lasted ~30 min.

6-2d Behavioural Testing

On days 1, 3, 5, 7, 14, 21, and 28 after surgery, we assessed all rats on the horizontal ladder, forelimb use asymmetry (cylinder), adhesive tape removal, beam walking, and forelimb inhibition (swim) tests (Fig. 1). Neurological deficits were

evaluated on a Neurological Deficit Scale (NDS) at these times and on days 2, 4, and 6 post-surgery. Skilled reaching was assessed in the staircase and single pellet tests on days 7 – 10 and 21 – 24.

Horizontal-Ladder Walking Test

Rats were videotaped crossing the middle 0.5 m segment of a 1 m long horizontal ladder with variably spaced rungs ranging from 3 to 5 cm. The total number of steps and number of slips made with each limb was recorded over 4 trials per test day. A detailed analysis of stepping was performed for baseline, day 7, and day 28[26]. Briefly, for each limb, each step was rated on a 7 point foot fault scale: (0) total miss, (1) deep slip, (2) slight slip, (3) replacement, (4) correction, (5) partial placement, and (6) correct placement.

Forelimb Use Asymmetry Test (Cylinder)

Rats were placed in a transparent cylinder (20 cm diameter, 45 cm high) for 10 minutes and videotaped from below. Spontaneous forelimb use during rearing movements, wall exploration, and landings was analyzed. Briefly, a push-off is the independent use of either forelimb or simultaneous use of both when rearing. Wall exploration is the initial placement of a forelimb on the wall and contact during lateral movements. A landing is the use of either limb (or both) to land after rearing. Rats that made fewer than 6 independent wall touches were excluded from analysis, as this was considered too few to be a reliable measure of movement frequency. Independent forelimb use was expressed as[27]:

[Number of contacts with contralateral limb + ½ both / (ipsilateral limb use + contralateral limb use + both)] × 100.

Adhesive Tape Removal Test

Adhesive dots (0.64 cm diameter, Avery; Pickering, Ontario, Canada) were placed on the medial aspect of the rat's forepaws. The order of placement (e.g., left then right) was randomized, and the paws were touched simultaneously before the rat was returned to its cage. The time taken to remove the adhesive dot from each paw was recorded on 3 trials. An asymmetry score[28] for this sensory or attention impairment (neglect) was calculated as:

Σ (Time to remove dot from contralateral paw – time to remove dot from ipsilateral paw) / number of trials.

Beam-Walking Test

Rats were videotaped crossing the beam (1.10 m long; 3.20 cm wide), and hind limb use was analyzed according to Feeney[29]. Briefly, performance was graded as: 0 (rat fell off the beam within 10 s), 1 (rat remained on the beam for more than 10 s but could not place the affected limb on the beam), 2 (rat was unable to cross but could place the affected limb on the beam and maintain balance), 3 (rat traversed beam while dragging the affected limb), 4 (rat crossed the beam and placed the affected limb on the beam at least once), 5 (rat crossed with more than 50 % foot slips with the affected limb), 6 (rat crossed with fewer than 50 % foot slips with affected limb), or 7 (rat crossed with 2 or fewer foot slips). Performance each test day was expressed as a median score of 3 trials.

Neurological Deficit Scale (NDS)

Neurological deficits were repeatedly measured[30]. Tests included: (1) spontaneous circling, graded from 0 for no circling to 3 for continuous circling; (2) hind limb retraction, graded from 0 for immediate replacement to 3 for no retraction after the limb was displaced laterally; (3) bilateral forepaw grasp, graded from 0 for normal grasping to 3 for a rat unable to grasp the bar; (4) contralateral forelimb flexion, graded from 0 for uniform extension of forelimbs to 2 for full wrist flexion and shoulder adduction when the rat was lifted by the base of the tail, and (5) beam walking ability, graded from 0 for a rats that readily crossed the beam to 3 for a rat unable to stay on the beam for more than 10 s. Scores for each component were added for a maximum of 14 (greatest impairment).

Staircase Test

Rats were food deprived to 90% of their free-feeding weight 4 days prior to testing in the skilled reaching tests. Rats received two 15 minute trials separated by ~ 4 hours on each of the 4 days of testing. We analyzed the number of pellets successfully retrieved out of a maximum of 21 per side.

Single Pellet Test

The number of pellets successfully retrieved (out of 20) was recorded on each test day. Performance was videotaped on days 10 and 24, and the movement components of 3 successful reaches for each rat were analyzed as described earlier.

Forelimb Inhibition Test (Swim)

Three trials were videotaped as each rat swam directly to the platform. Rats that swam along the wall or those that did not reach the platform after a maximum of 10 trials

were excluded from analysis. The number of strokes made with each forelimb was counted and expressed as an asymmetry score for forelimb inhibition.

6-2e Histology

Thirty days following surgery, rats were euthanized with an overdose of sodium pentobarbital (80 mg/kg) and transcardially perfused with 0.9% saline and then 10% neutral buffered formalin. Forty μm sections were taken every 200 μm, starting at +1.7 from Bregma and ending at –4.8 mm to Bregma. Sections were then stained with cresyl violet. The volume of lesion (e.g., cavity, cellular debris) plus atrophy (e.g., ventriculomegaly) was calculated manually using Scion Image J 4.0 (Scion Corporation, Frederick, MD, U.S.A.) as follows and as routinely done[24, 25]:

> Volume of tissue lost = remaining volume of normal hemisphere – remaining volume of injured hemisphere.
>
> The 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.

6-2f Statistics

All behavioural and histological analyses were done by experimenters blind to group identity. Most data were analyzed by ANOVA and subsequent group comparisons (usually Fisher LSD tests). In cases of a significant Levene's test (i.e., heterogeneous variance) we used independent samples t-tests (equal variances not assumed). For non-parametric data (e.g., rating scales) we used the Kruskal Wallis test followed by Mann Whitney U comparisons. Chi-square tests were used to assess dropout rate. Regression

analyses determined which combination of behavioural tests predicted lesion volume the best. For all ICH rats, we ranked lesion volume and performance on each test (from best to worst), and used Spearman's rank to determine how each test independently related to histology. Lesion volume was assessed by ANOVA and conservative Scheffe post-hoc tests to help ensure that lesion volumes were truly different. However, we used the LSD test for multiple comparisons with the behavioral data in order to maximize our chances of detecting significant functional differences among our groups. This is also why we used relatively large group sizes and repeated testing (i.e., to improve statistical power).

6-3 Results

6-3a Lesion Volume

No mortality occurred in this study. One SHAM rat had damage in addition to the needle tract, and was thus excluded from analysis. This was likely due to a needle-induced hemorrhage. A one-way ANOVA ($p < 0.001$) followed by Scheffé tests showed significant differences in injury among all groups ($p \leq 0.044$; Fig.2).

6-3b Behavioral Assessment

Baseline performance in each test was similar among all groups (data not shown). The time needed to conduct training, testing and analysis for each behavioral test is given in Table 1.

Horizontal Ladder Walking Test

Many ICH rats failed to cross the ladder on days 1 (60.0 %; $p = 0.023$) and 3 (35.6 %; $p = 0.001$), and thus data for these days were not analyzed further. One-way ANOVAs revealed significant effects of GROUP at all test days for the contralateral forelimb ($p \leq 0.007$; Fig. 3). The percentage of slips versus SHAM was greater in the

ICH groups at all times ($p \leq 0.026$), except for MILD on day 14 ($p = 0.177$), and MODERATE on day 7 ($p = 0.058$). Large differences in error rates were sometimes detected among ICH groups (e.g., between MILD and SEVERE groups on days 14 to 28; $p \leq 0.036$); however, smaller effects were not significant (e.g., between MILD and MODERATE groups; $p \geq 0.135$). Analysis of the contralateral hind limb revealed significant GROUP effects at each test day ($p \leq 0.018$). The MILD group made significantly more errors than the SHAM group on days 21 ($p = 0.019$) and 28 ($p = 0.008$) only, whereas the MODERATE and SEVERE groups consistently made more errors than SHAM ($p \leq 0.031$). Differences in error rates were not always detected between the MILD and MODERATE or MODERATE and SEVERE groups; however, the SEVERE group made significantly more errors than the MILD group on days 5, 7, and 21 ($p \leq 0.017$). Ipsilateral forelimb and hind limb error rates were not significantly different among groups ($p \geq 0.113$).

A more detailed analysis of each step (graded from 0 for a total miss, to 6 for correct placement) on days 7 and 28 revealed that the ICH groups made more slight slips with the contralateral forelimb compared to the SHAM group on day 7 and day 28 ($p \leq 0.018$ and $p \leq 0.024$, respectively; data not shown). Furthermore the MILD group made more replacements ($p = 0.023$), and the SEVERE group made more deep slips ($p = 0.041$) and fewer correct steps ($p = 0.034$) compared to the SHAM group on day 7. There were no differences among the ICH groups ($p \geq 0.082$) on day 7, but the SEVERE group made more slight slips than the MILD ($p < 0.001$) and MODERATE groups ($p = 0.035$) on day 28. Data for the contralateral hind limb were similar (not shown). The ipsilateral forelimb and hind limb steps were similar among groups ($p \geq 0.061$, not shown).

Forelimb Use Asymmetry (Cylinder) Test

Data from 15 test sessions (out of 472) were excluded because rats made fewer than 6 independent wall touches during the videotaped session. The dropout was not significantly different among groups ($p = 0.090$). For push-off and landing, differences in independent contralateral forelimb use were rarely detected (data not shown). Contralateral forelimb use during wall exploration revealed a significant effect of GROUP at all times ($p \leq 0.014$; Fig. 4). All ICH groups used their contralateral forelimb less than SHAM ($p \leq 0.003$), except for the MILD group on day 3 ($p = 0.111$), and the SEVERE group on days 1 ($p = 0.778$) and 3 ($p = 0.184$). Interestingly, the SEVERE group used their contralateral forelimb significantly more than MILD ($p = 0.028$) and MODERATE groups ($p = 0.008$) on day 1 and more than the MODERATE group on day 3 ($p = 0.040$). This was likely due bilateral deficits in the SEVERE group. Otherwise significant differences among the ICH groups did not occur ($p \geq 0.071$).

Adhesive Tape Removal Test

There were significant GROUP main effects on all days ($p \leq 0.025$, Fig. 5). Differing rates of recovery were detected with this test. For example, the MILD group was impaired vs. SHAM rats only on days 1 and 3 ($p \leq 0.019$), whereas impairments were detected in the MODERATE group until day 21 ($p \leq 0.041$ vs. SHAM). Compared to SHAM or MILD groups, the SEVERE group took longer to remove the dot on the contralateral forelimb on all days ($p \leq 0.030$) except day 1 ($p \geq 0.065$). Large differences among the ICH groups (e.g., between MILD and SEVERE groups) were statistically significant on all days ($p \leq 0.030$) except day 1 ($p = 0.065$). Smaller effects were rarely

detected. For instance, the MODERATE and SEVERE groups were different only on days 14 and 21 ($p \leq 0.041$) and not on other days ($p \geq 0.110$).

Beam Walking Test

There was a significant effect of GROUP on all test days ($p \leq 0.018$) except day 28 ($p \leq 0.072$; Fig. 6). Differences in contralateral hind limb errors were usually not detected among ICH groups with the exception of the MILD and SEVERE groups on day 5 and 7 ($p \leq 0.023$). Interestingly, the rate of recovery varied among the ICH groups. For example, the MODERATE and SEVERE groups were persistently impaired versus SHAM until day 21 ($p \leq 0.014$) and day 28 ($p \leq 0.033$), respectively, whereas the MILD group had fully recovered by day 7 ($p \geq 0.134$ vs. SHAM).

Neurological Deficit Scale

Each ICH group had significant neurological impairments (higher NDS) from 1 to 28 days after ICH (vs. SHAM; $p \leq 0.001$; Fig. 7). Small group differences were rarely detected. For instance, the MODERATE group had significantly greater deficits than the MILD group only on day 5 ($p = 0.037$), and differences between the SEVERE and MODERATE group were detected only on days 5 and 7 ($p \leq 0.029$). However, the MILD and SEVERE groups were different from each other on days 1, 2, 3, 5, 6, 7, and 21 ($p \leq 0.024$).

Staircase Test

Two rats were excluded from this test because they failed to meet the criterion during training. A repeated measures ANOVA for each test session revealed significant GROUP ($p \leq 0.001$) and DAY ($p \leq 0.005$) main effects. All groups retrieved more pellets over days. Each ICH group retrieved significantly fewer pellets than SHAM animals on

days 7 – 10 ($p < 0.001$; Fig 8), and ICH groups were significantly different from each other ($p \leq 0.040$). Data were similar for days 21 – 24, however, the MILD and MODERATE groups could not be distinguished from each other ($p = 0.058$). Significant GROUP main effects ($p < 0.001$) were also detected for the number of pellets retrieved with the ipsilateral forelimb. On days 7 – 10, all groups were significantly different from each other ($p \leq 0.035$) with the exception of MILD and SHAM groups ($p = 0.502$). However, by days 21 – 24, only the SEVERE group was impaired ($p < 0.001$ vs. SHAM). The SEVERE group was also significantly different from the other ICH groups at this time ($p \leq 0.025$).

Single Pellet Test

Fifty-three percent of the MODERATE group and 67.7 % of the SEVERE group did not reach with the contralateral forelimb in the single pellet test on days 10 and 28 ($p = 0.005$). Instead they reached with their initially non-dominant limb (i.e., they switched limb preference) or did not reach at all. Thus, only the MILD and SHAM groups were analyzed for reaching success and quality of reaching. A 3-way ANOVA revealed significant GROUP ($p < 0.005$) and DAY ($p = 0.029$) main effects. The MILD group retrieved fewer pellets (vs. SHAM), and both groups retrieved more pellets on subsequent days during each test session. Qualitative rating of reaching movements for successful reaches demonstrated that both aim ($p = 0.023$) and grasping ($p = 0.021$) were impaired in the MILD group (vs. SHAM) at day 10. However, only the grasping deficit persisted until day 24 ($p = 0.008$; data not shown).

Swim Test

Data from 50 test sessions (out of 472) were excluded because some rats failed to swim directly to the platform (e.g., swam along the walls or did not reach platform). Most of this occurred on days 1 and 3; however, the dropout rates were not significant ($p \geq 0.062$). There was a significant effect of GROUP on all days ($p \leq 0.027$), but day 1 ($p = 0.058$). The ICH groups used their contralateral forelimb more frequently than the SHAM rats on most days (i.e., had significantly higher difference scores; $p \leq 0.029$). However, impairments (vs. SHAM) were not detected in the MILD group on day 28 ($p = 0.102$), the MODERATE group on days 14 and 21 ($p \geq 0.078$), and the SEVERE group on day 14 ($p = 0.051$). No significant differences occurred among any of the ICH groups at any time ($p \geq 0.086$; Fig. 9).

6-3c Composite Behavioural Analyses

To assess overall performance, rats were ranked from best to worst on their average or median performance across test days for each test (e.g., mean pellets retrieved with the contralateral forelimb in staircase test). These ranks were averaged, and an ANOVA of the mean composite scores revealed a GROUP main effect ($p< 0.001$) and significant differences among all groups was seen with posthoc analysis ($p \leq 0.032$; Fig. 10a). In general, rats with smaller lesions ranked better than ones with large lesions. The overall ranked performance across all tests correlated well with the ranked volume of injury ($r = 0.718$, $p < 0.001$; Fig. 10b; also see Table 2 for r values for each test). Similar findings were found with correlations between actual lesion volume and performance for individual tests or the composite score (i.e., Pearson r values; data not shown). Regression analysis revealed that the combination of behavior tests that best predicted histology depends on the time of assessment. For example, at day 7 the adhesive tape

removal, horizontal ladder and cylinder tests had the highest relationship to lesion volume. At day 28, the staircase, cylinder, adhesive tape removal tests and NDS strongly related to histology. When overall performance was considered, only the adhesive tape removal test and NDS significantly predicted lesion volume in the multiple regression analysis.

6-4 Discussion

Prior to clinical investigation, prospective cytoprotectants should be shown to improve functional outcome in animal stroke models (e.g., rat). Accordingly, there is a need for tests that detect stroke damage as well as a cytoprotective effect. Presently, we used a range of functional tests (e.g., walking, skilled reaching) to determine whether they could detect a subcortical ICH lesion, and if testing could distinguish among gradations in lesion size akin to significant cytoprotective effects. Our results show that while behavioral testing easily detected ICH-induced subcortical injury, only a few tests frequently distinguished among groups and strongly correlated to lesion size, and of these, none consistently differentiated among all ICH groups. Therefore, we urge investigators to use a battery of tests, especially if moderate treatment effects are expected.

There are several issues to consider when selecting behavioral tests for rodent stroke studies. First and foremost, deficits depend on the location of injury, and thus the tests should be chosen with this in mind. For instance, damage to the dorsomedial striatum disrupts locomotor activity, whereas a more lateral lesion affects skilled motor control[31, 32]. Accordingly, if a certain behavior were controlled by only one sub-section of a damaged site, a larger lesion affecting other portions of that structure would not be

expected to produce greater impairment. An ICH, however, often crosses functional boundaries as presently seen (e.g., damage to the corpus callosum, internal capsule and striatum). Thus, a broad range of deficits (e.g., in skilled reaching, forelimb inhibition, and walking) such as occurs after an ICH is more likely to be detected using a battery of tests, rather than one or two tests. Additionally, behavioral deficits might only occur when injury has reached a critical threshold and then remain unchanged. In our study, several tests (e.g., cylinder and swim tests) were effective "lesion detectors," but did not distinguish among gradations in injury. The utility of testing also depends on timing. For instance, ICH rats showed maximal deficits soon after ICH in the beam test only to later show an apparently complete recovery in all groups. In addition, many rats did not cross the horizontal ladder during the first week after ICH and many MODERATE and SEVERE rats refused to reach with their impaired limb in the single pellet task, even weeks after the ICH. Finally, cytoprotection studies must take into account whether food deprivation is needed as it may be contraindicated if the stroke or treatment produces lasting reductions in body weight and appetite.

Given these concerns, it makes sense to broadly test behavior after ICH. However, during the early (1 week) post-ICH period we recommend the NDS owing to its ability to distinguish among groups reasonably well across a range of injuries, in addition to the simple and quick nature of testing and data analysis. If long-term deficits are sought, then the staircase test is highly recommended. While the data are easy to analyze, the test is time consuming (e.g., training is recommended) and it requires food deprivation. Neither the NDS nor staircase test, however, consistently distinguished among all groups. Therefore, other tests such as the tape test should also be used. It

should be noted that the time required for each of these tests can be reduced by simply reducing the number of testing sessions from the large number we used. The use of detailed analyses of skilled reaching and walking did not produce data that distinguished groups any better than endpoint measures (e.g., reaching success). Given the time required for these analyses, we do not recommend these procedures for routine cytoprotection studies, which commonly use large numbers of animals. However, these tests are appropriate for studies aimed at identifying whether rats truly recover or compensate, which at some point is important in cytoprotection studies. Finally, it was clear that groups were easily distinguished by a battery of tests more so than with any particular test. Thus, multiple tests should be used in cytoprotection studies.

Our findings suggest that cytoprotection studies that have relied upon a single test might have missed important treatment effects, especially if they were relatively small. However, studies have used tests, such as NDS, after ICH and have found functional improvements with experimental cytoprotectants. Interestingly, in several cases behavioral improvements occurred despite the absence of discernable histological protection[30, 33]. We suspect that such treatments are acting, at least in part, on residual tissue (e.g., dendritic branching and number of synapses), and thus are not simply acting to reduce tissue loss via attenuating cell death. Indeed, it is possible that such treatment strategies are, at present, a better approach to improving functional outcome than simply reducing lesion size as recently found in a study examining hypothermia and rehabilitation treatments after ICH in rats[34]. Alternatively, beneficial effects on one or two tests may not necessarily occur with other tests. Likewise, it is possible that significant treatment effect with a NDS may be due to one sub-component and not others.

There are several limitations to this study. First, we did not assess performance on cognitive tests, or on all of the sensory and motor tests (or their variations) known to be sensitive to striatal injury (e.g., corner turn test[15] and rotarod[16]). Such tests might be better at discerning gradations in injury. Lasting sensorimotor impairments, however, would likely confound cognitive tests and we had to limit the already extensive amount of sensorimotor functional testing. Second, we assessed performance repeatedly on all tests and it is possible that this inadvertently acted as rehabilitation, thus enhancing the degree of "spontaneous" recovery and thereby lessening group differences. The fact that group trends were largely the same throughout the testing periods, however, argues against this explanation. Third, the presence of bilateral deficits (e.g., as seen with the staircase test) may have affected our results with the cylinder, swimming and adhesive tape tests. For example, the SEVERE group was initially better than the MILD and MODERATE groups in the cylinder test. Given the severity of the insult, it is likely that the ipsilateral limb of SEVERE rats was affected. Therefore, deficits may have been masked by bilateral impairments. Accordingly, such difference scores must be interpreted cautiously. Fourth, we did not determine the utility of these tests in the autologous whole blood model of ICH. Further study is clearly needed in this model to determine if treatment effects can be reasonably determined with these tests. Fifth, we did not relate behavior to other important endpoints (e.g., edema) that may influence performance[15]. Sixth, choice of statistical analysis clearly influences outcome. We used a stringent post-hoc test to show that the lesion volume was significantly different among ICH groups, whereas no corrections were made for multiple comparisons with the behavioral tests. Significant differences in functional outcome infrequently occurred between the smaller

gradations in injury (e.g., MILD vs. MODERATE), and more stringent post-hoc testing would only exacerbate this. We have also noticed that many stroke investigators inappropriately analyze ordinal data (e.g., NDS) by using ANOVA as well as presenting this data as mean and standard deviation or error. Finally, use of larger group sizes may improve statistical power sufficiently to detect group differences. However, we used approximately 15 rats per group, which is more than that typically used in this field where group sizes are often less than 10. Furthermore, the overlap in lesion sizes among the ICH groups realistically reflects treatment effects seen in the literature. Thus, cytoprotection studies, as currently done, are not likely to consistently detect small functional differences.

Effective pre-clinical testing of putative cytoprotective agents for brain injury requires appropriate functional evaluation in addition to quantification of brain injury[5-9], especially because the latter is necessarily incomplete. Behavioral tests should be validated for the type of insult produced, and must be sensitive to injury as well as the effects of treatments. Most experiments test whether a behavioral test is sensitive to injury, but fail to examine whether it can distinguish among lesion sizes that occur with a cytoprotective intervention. Indeed, many of the tests we used did not consistently distinguish among sizeable gradations in injury, but did detect a lesion. Therefore, we recommend using a battery of tests (e.g., staircase, NDS and tape tests) sensitive to a range of deficits over several testing sessions (e.g., within first week, near one month). Furthermore, we strongly encourage investigators to evaluate potential tests across a range of insult severities in their particular model because other factors will impact the utility of testing (e.g., strain, age, gender)[35-37]. Also, when designing an appropriate test

battery for cytoprotection studies, researchers should consider the type of injury, timing of testing, the time required to complete training and testing, the limitations of tests, and the sample size needed to detect differences in outcome. Only with such a battery might we truly identify effective cytoprotection in rodent models that then hopefully pass clinical scrutiny.

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Table 6-1: Estimated time needed to conduct training, testing, and analysis for each behavioral test. Numbers listed represent the time required for one assessment per rat, except for the staircase and single pellet tests, which require multiple sessions. The number in parentheses denotes time needed for kinematic analysis of movements. Estimates do not include time needed for weighing and feeding rats during periods of food deprivation, data entry, statistical analyses, etc.. In the staircase test, multiple rats can be trained and tested concurrently.

Behavioral Test	Training	Testing	Analysis
Adhesive Tape	5 min	5 - 15 min	Minimal
Staircase	40 min × 15 days	40 min × 4 days	Minimal
Single Pellet	15 min × 15 days	15 min × 4 days	Minimal (5min)
NDS	15 min	10 min	5 min
Cylinder	10 min	10 min	20 min
Ladder	5 min	5 min	10 min (20 min)
Beam	10 min	5 min	5 min
Swim	25 min	5 min	5 min

Table 6-2: Relationship (Spearman's rho) between ranked lesion volume (at 30 days) and early (day 7), late (day 28), or overall ranked performance on each behavioural test for all ICH rats. Number in parentheses denotes *p* value. See Figures 3 to 10 for scatter plots of ranked performance and ranked injury volume. This type of analysis was not done for the beam walking test, as the Spearman correlation was invalidated by a high number of similar scores (32/45 rats).

Behavioral Test	Early	Late	Overall
Adhesive Tape	0.773 (< 0.001)	0.459 (0.002)	0.770 (< 0.001)
Staircase	0.679 (<0.001)	0.585 (<0.001)	0.646 (<0.001)
NDS	0.421 (0.004)	0.354 (0.017)	0.463 (0.001)
Ladder	0.326 (0.033)	0.401 (0.008)	0.415 (0.005)
Swim	0.301 (0.050)	0.259 (0.086)	0.312 (0.037)
Cylinder	0.272 (0.078)	0.367 (0.014)	0.278 (0.065)

Figure 6-1: Behavioral testing (days relative to surgery) schedule. During testing, all rats were assessed on each of the behavioural tests. See Methods for description of tests. Timeline for behavioural training (pre-ICH) is not shown.

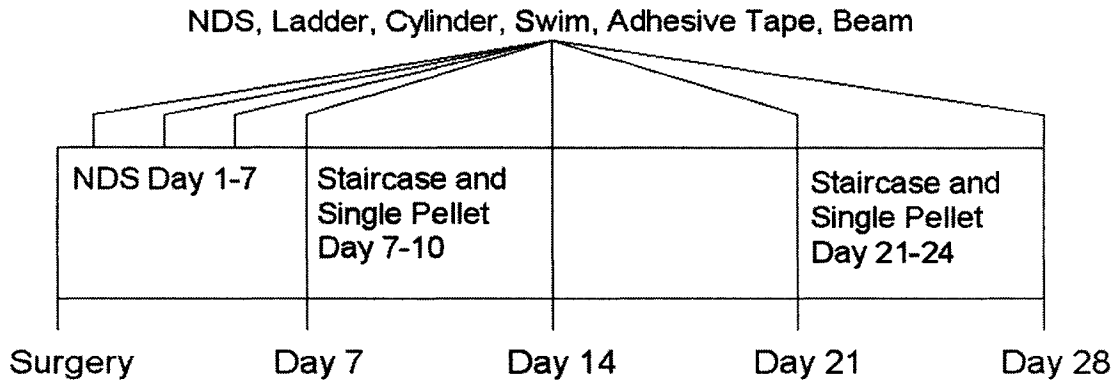


Figure 6-2: Volume of tissue lost (mean ± SEM) at 30 days after ICH / SHAM surgery (A). All groups were significantly different from each other. Photomicrographs represent a typical lesion (e.g., cavity, cellular debris, and ventriculomegaly) in the MILD (B), MODERATE (C), and SEVERE (D) groups. The hematoma was nearly completely reabsorbed by 30 days making the demarcation of injury easy. Damage occurred primarily to the striatum (anterior to posterior) but sometimes included the globus pallidus, corpus callosum, and thalamus.

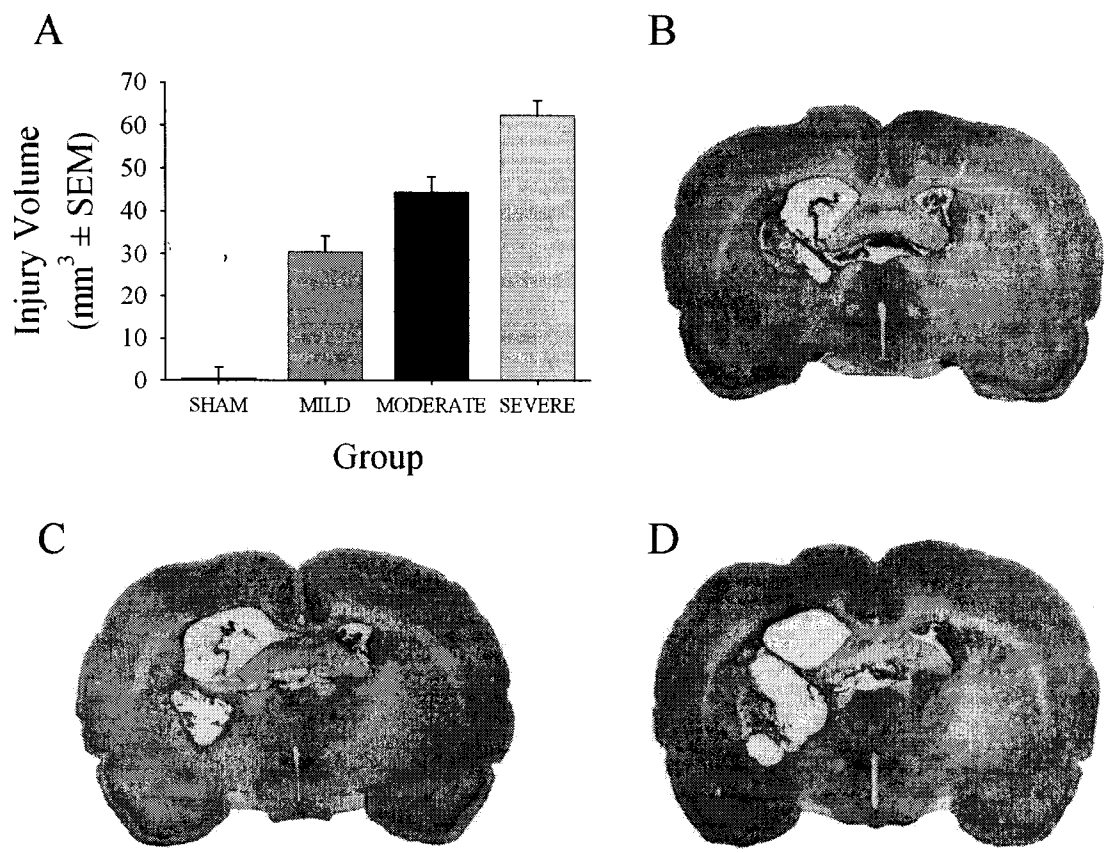


Figure 6-3: Contralateral forelimb error rate (% slips through bars) in the horizontal ladder-walking test from baseline (BL) to 28 days post ICH / SHAM operation (A). Data for days 1 and 3 are not shown due to a significant dropout rate difference among the groups (some ICH rats would not cross the ladder). Differences among ICH groups were frequently detected. The relationship between ranked slip rate and ranked lesion size for ICH rats was statistically significant (B; Table 2).

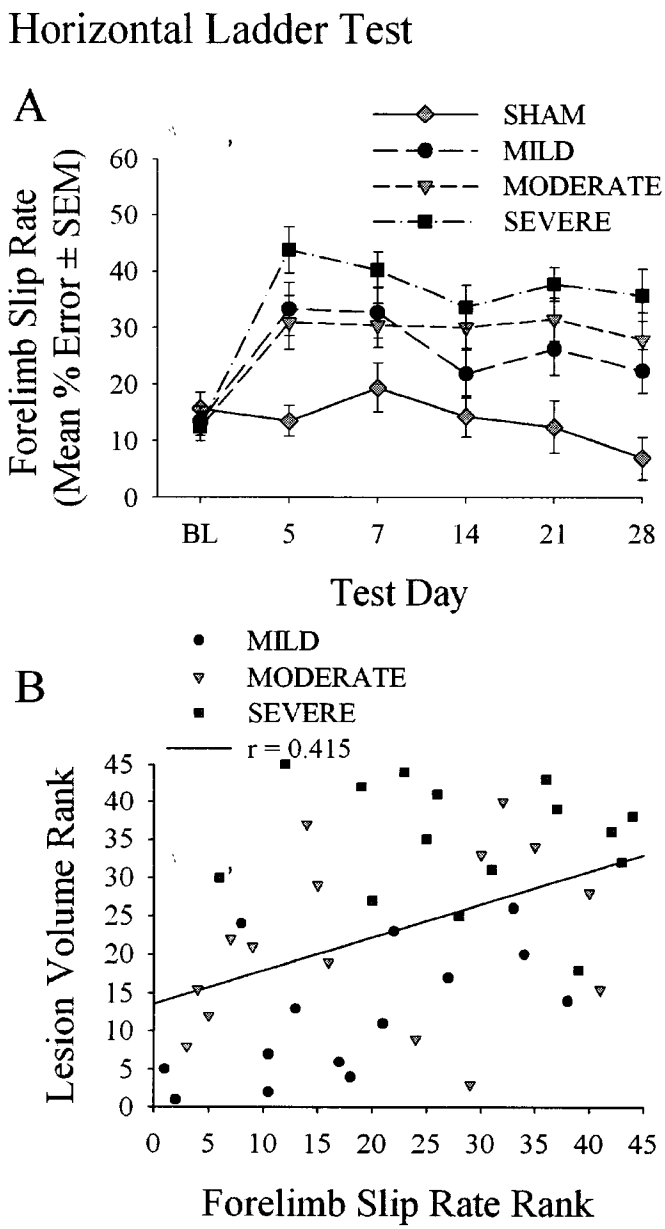


Figure 6-4: Spontaneous contralateral forelimb use [number of contacts with the contralateral limb + ½ both / (ipsilateral + contralateral limb use + both)] during exploration of walls in the cylinder test (A). All groups were impaired vs. SHAM, but differences among ICH groups were not detected. Relationship between contralateral limb use (ranked performance) and ranked lesion volume for ICH rats (B).

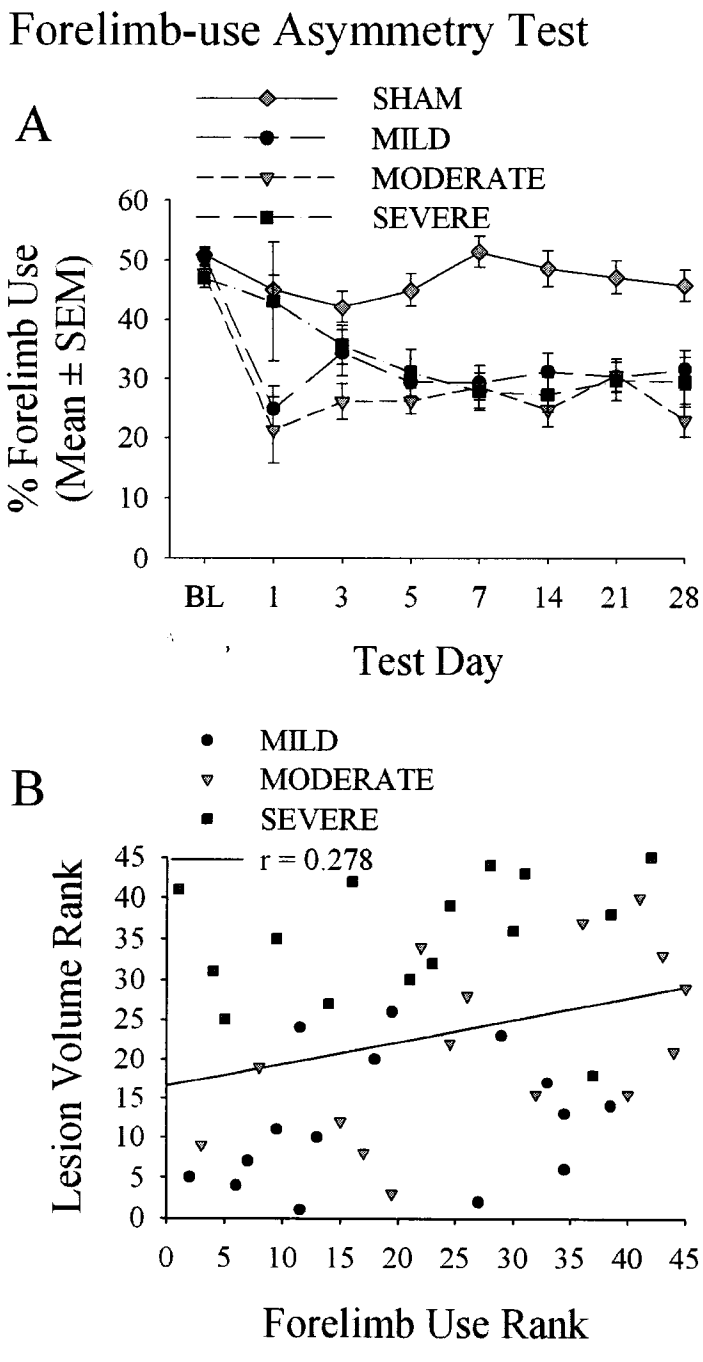


Figure 6-5: Difference score (seconds; time to remove dot from contralateral forelimb – time to remove dot from ipsilateral forelimb) in the adhesive tape removal test from baseline (BL) to 28 days after ICH / SHAM operation (A). Differences among ICH groups were frequently, but not consistently detected. The relationship between ranked score and ranked brain injury for ICH rats was significant (B; Table 2).

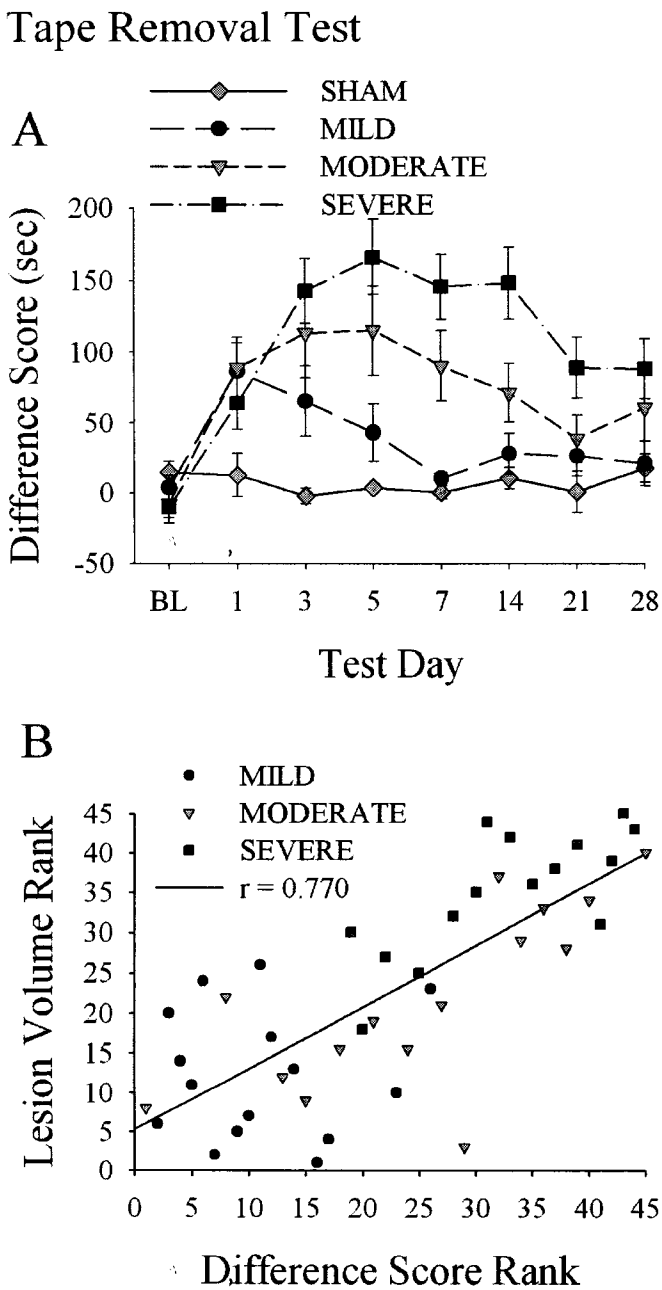


Figure 6-6: Contralateral hind limb deficit score (median group score) in the beam-walking test. Differing rates of recovery were detected.

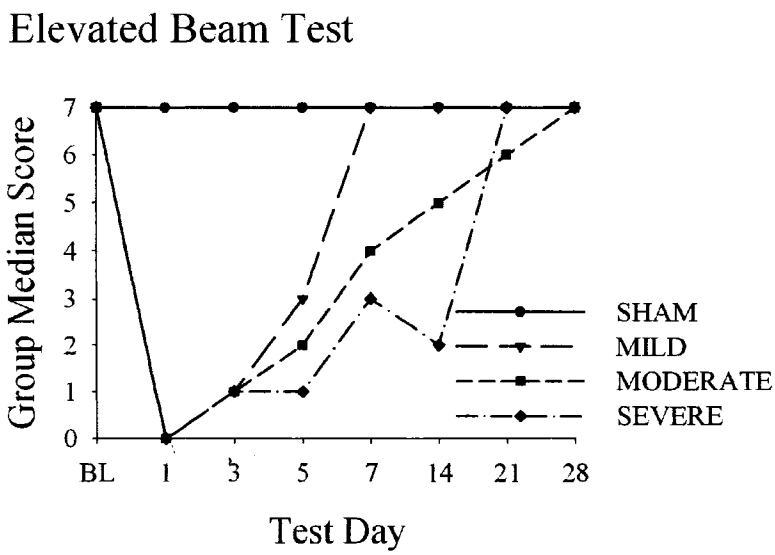


Figure 6-7: Neurological impairments (median NDS score) from baseline (BL) to 28 days after ICH / SHAM surgery. A score of 14 indicates maximum impairment. Differences among groups were often detected. The correlation between ranked NDS and ranked brain injury is significant (B; Table 6-2).

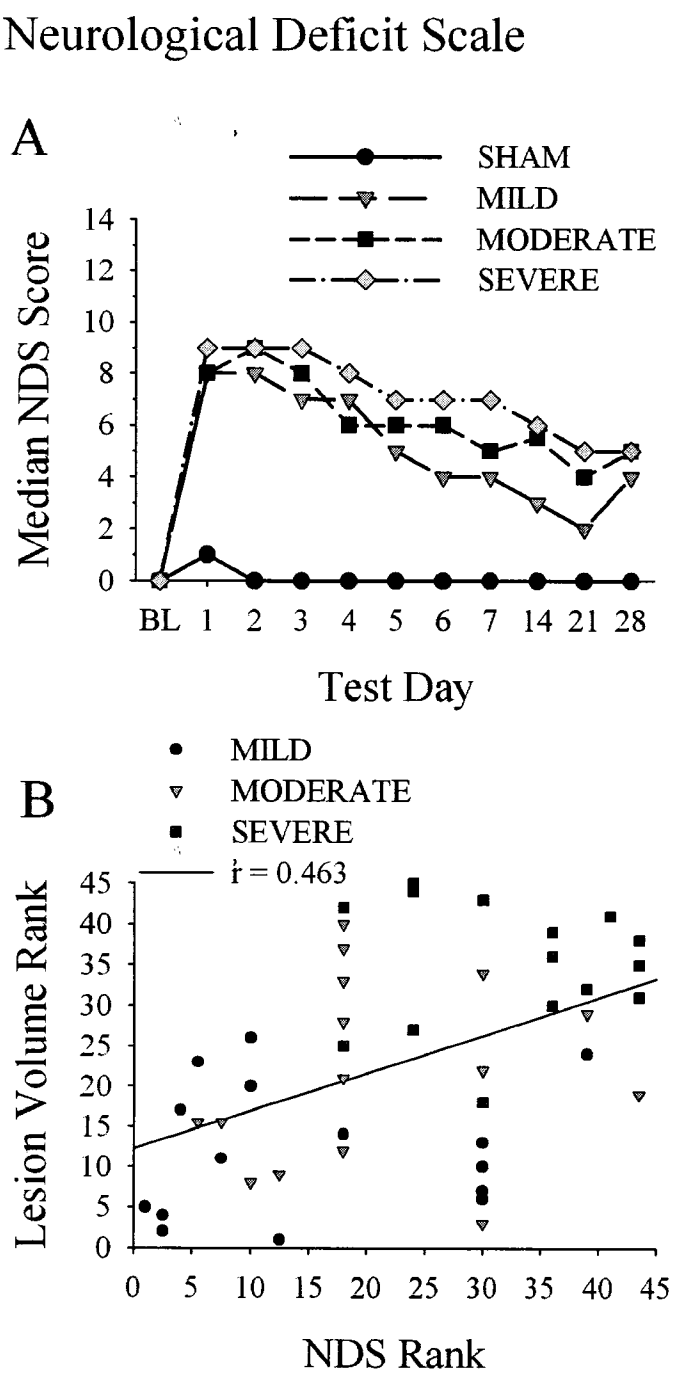


Figure 6-8: Number of pellets retrieved with the contralateral forelimb in the staircase test for the baseline (BL) period and on days 7 – 10 and 21 – 24 after ICH / SHAM operation. All groups were significantly different from each other on days 7 –10, however the MILD and MODERATE groups could not be distinguished on days 21 – 24. A significant relationship occurred between ranked reaching performance and ranked lesion volume among ICH groups (B; Table 6-2).

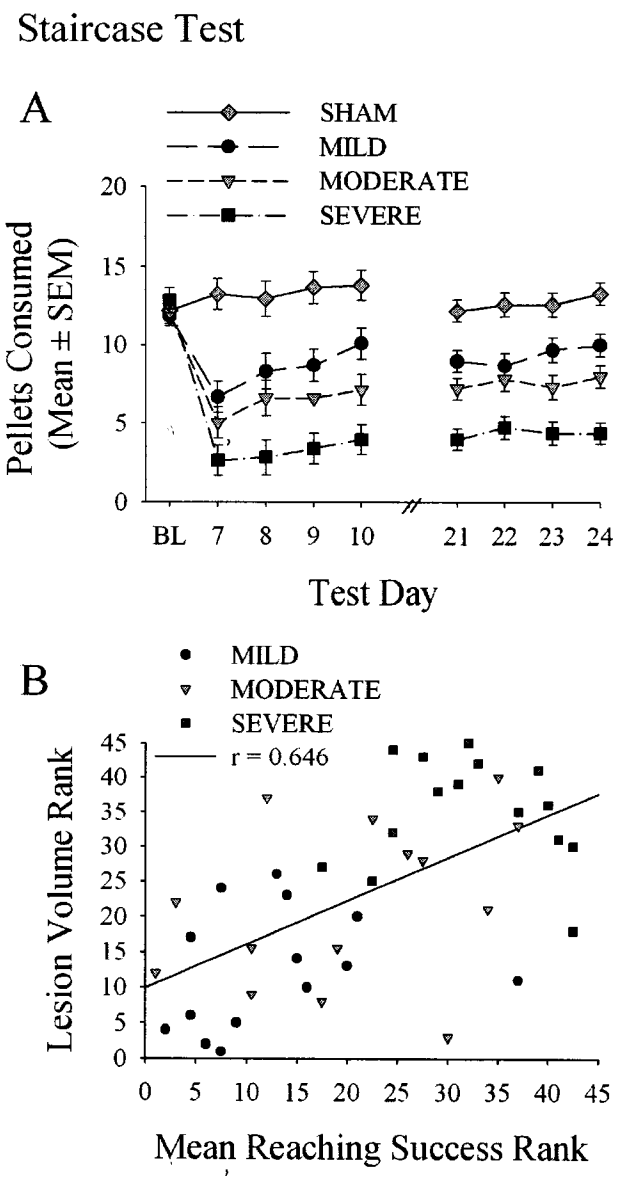


Figure 6-9: Difference score [(number of strokes with contralateral forelimb – number of strokes with the ipsilateral forelimb) / number of trials] in the forelimb inhibition (swim) test from baseline (BL) to 28 days after ICH / SHAM operation. Statistically significant differences among ICH groups were not found. However, the relationship between ranked performance on this test and the ranked brain injury for ICH rats reached statistical significance (B; Table 6-2).

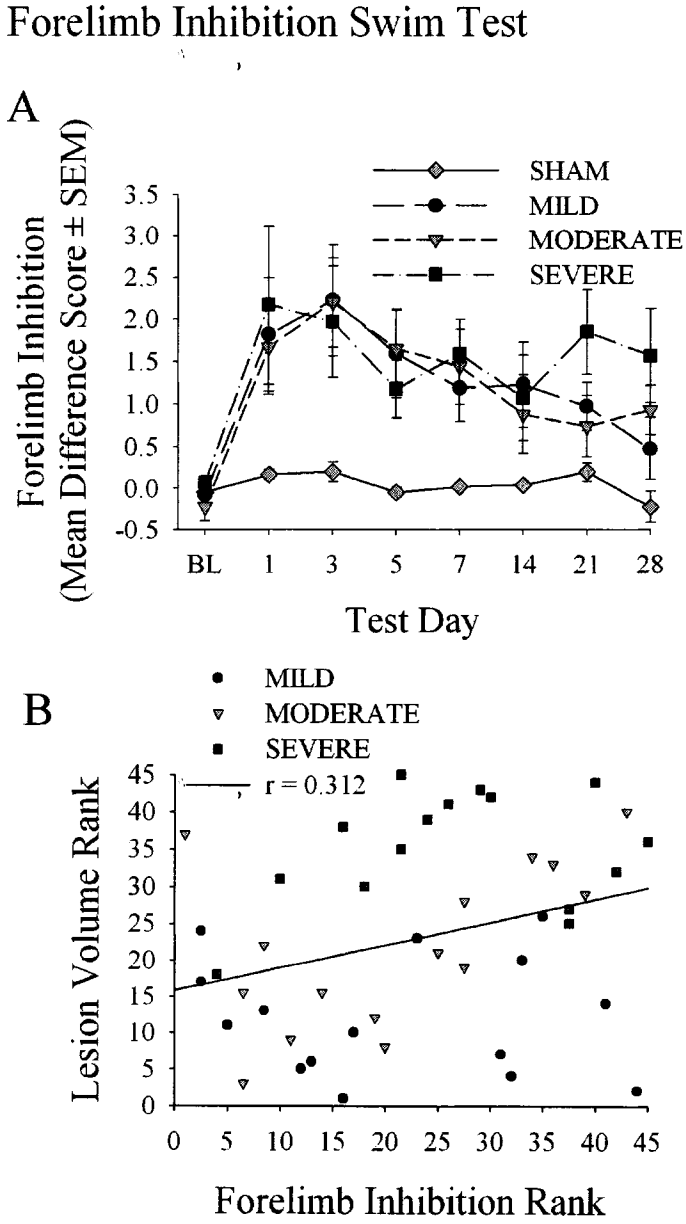
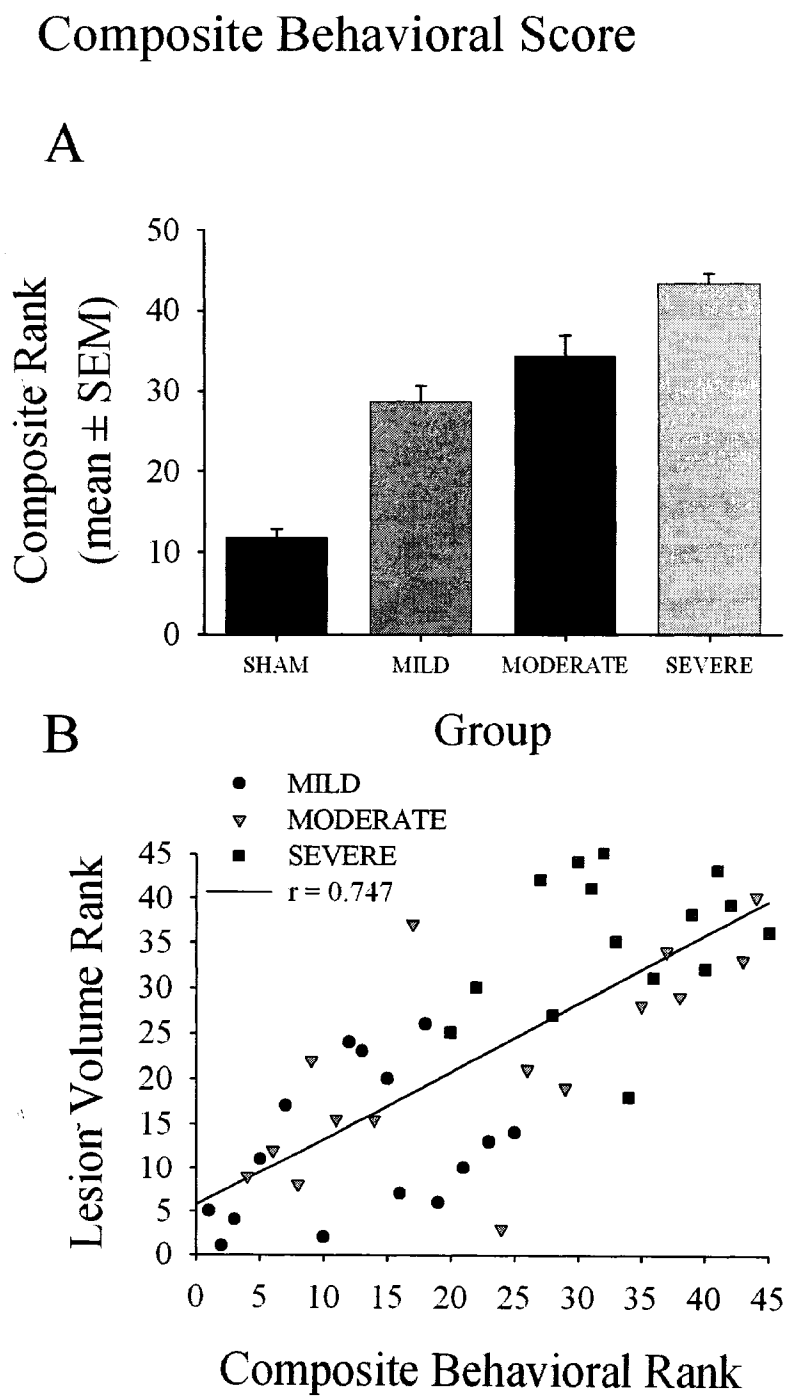


Figure 6-10: Composite behavioural score (mean rank ± SEM) for overall performance in all tests (A). All groups were significantly different from each other. There was a strong correlation between behavioural performance and ranked lesion volume in ICH rats (B).



Chapter 7: Skilled reaching impairments follow intrastriatal hemorrhagic stroke in rats.

A version of this chapter is under review: MacLellan, C.L., Gyawali, S., and Colbourne, F. *Behavioural Brain Research.*

7-1 Introduction

Intracerebral hemorrhage (ICH) is one of the deadliest types of stroke and survivors (~ 60 % of ICH patients) are often left with significant disability [1,2]. For instance, arm and digit movements necessary for reaching and grasping are persistently impaired in stroke patients. [3,4] Likewise, motor system injury in rodents, including damage to the lateral striatum, [5,6] impairs skilled reaching ability. Over the past decade, there has been a surge of studies identifying the mechanisms of brain injury after ICH and in testing prospective therapies [7]. Although many experimental ICH studies report promising therapeutic results, there has been little translation to the clinic [7]. This failure is likely due to limitations in both experimental and clinical practices, which have been identified for ischemia [8,9] and ICH studies [7]. In particular, experimental studies often fail to use long survival times and do not comprehensively assess behavioural outcome. Given that functional recovery is the endpoint of greatest clinical concern, it makes sense that recovery should be rigorously assessed in preclinical studies [10-12].

Researchers have examined many sensory and motor tests in rodent stroke models [13-15]. An ideal test should not only detect the presence of a lesion, but must also detect therapeutic effects. Recently, we [16] characterized neurological deficits after ICH induced by striatal infusion of bacterial collagenase, which causes bleeding by disrupting cerebral microvasculature [17]. Although the majority of the tests (e.g., skilled reaching, walking, neglect) detected ICH injury, few distinguished among gradations in injury [16]. Interestingly, the staircase task of skilled reaching was one of the most useful tests for detecting ICH injury and moderate differences in lesion size. However, functional

performance on a battery of tests, rather than any single test, best predicted histological outcome one month after ICH.

An alternative rodent model of ICH, induced by infusing autologous blood into the brain [18], is routinely used to study of the pathophysiology of ICH (for review, see [19]) and in some cytoprotection studies (e.g., [20]). The infusion of blood, rather than collagenase, better mimics the single large bleed that occurs in most ICH patients [21]. Using this ICH model, Hua and colleagues [22] carefully identified a battery of tests sensitive to injury, tracked recovery over time, and examined the relationship between functional outcome and brain edema. Although each of their tests (corner turn test, forelimb use asymmetry (cylinder) test and forelimb placing test) detected ICH injury, marked recovery to pre-surgical levels occurred by one month in two of the tests. Similarly, our recent study [20] showed ICH rats near completely recover in behavioral tests (e.g., cylinder, ladder and staircase tasks) otherwise sensitive to focal ischemia [23-25] and collagenase-induced ICH [16]. These findings highlight the need to identify sensitive tests for this model of ICH, especially over the long term.

We hypothesized that whole blood ICH would persistently impair skilled reaching in rats. Therefore, we assessed skilled reaching ability over one month after ICH in the staircase [24] and single pellet reaching tasks [26]. Our goal was to characterize skilled reaching following ICH, and to identify behavioral tasks that could form part of a test battery for assessing ICH therapeutics.

7-2 Materials and Methods

7-2a Animals

Twelve male Sprague Dawley rats were used. Rats were obtained locally and weighed 200 - 250 g (~ 8 weeks old) at the start of the experiment. Rats were housed in groups of four throughout the experiment and maintained on a 12 h light/dark cycle (07:00 - 19:00 h). All procedures were approved by the Biological Sciences Animal Policy and Welfare Committee at the University of Alberta and were in accordance with the Canadian Council on Animal Care guidelines.

7-2b Skilled Reaching Tasks

Rats were concurrently trained to reach for food reward pellets (45 mg each, Bio-Serv, Frenchtown, NJ, USA) in the Montoya staircase task (Fig. 1A) and the single pellet reaching task (Fig. 1B). Starting 3 days prior to training, rats were food deprived to 90% of free feeding weight and weighed daily to maintain weight at this level. Food restriction took into account the natural gain in body weight during the training period. This prevented excessive weight reduction.

Staircase Task

Rats were trained in the staircase task, which measures independent forelimb skilled reaching ability [24]. Briefly, each rat is placed into a clear plexi-glass box (length: 30 cm, width: 6.8 cm, height: 12 cm) in which the rat rests on a central elevated platform with 7 stairs descending on each side. Each stair is baited with 3 food pellets. Pellets on the left stairs may be retrieved only using the left paw, whereas pellets on the right stairs must be obtained using the right paw. Rats were trained over 4 weeks (two 15 min sessions per day, 5 days per week) and were excluded from this test if they did not retrieve at least 9 pellets per side (out of a possible 21) on three consecutive days [16, 27]. The last 5 days of training were used to calculate baseline performance. We quantified

skilled reaching ability (% success) by recording the number of pellets retrieved with each forelimb (i.e., [number of pellets retrieved / 21] × 100).

Single Pellet Task

Rats were trained to retrieve food pellets in the single pellet task 5 days per week over 4 weeks. For the test, rats were placed in a clear plexi-glass box (length: 60 cm, width: 14 cm, height: 35 cm) and trained to reach through a 1 cm wide opening to retrieve a food pellet placed on the ledge in front of the opening [28]. Initially, pellets were placed in both wells and reaching was followed by immediately replacing retrieved or displaced pellets. Once rats displayed a paw preference and were reliably reaching (usually within the first few days), the pellet was placed in the well contralateral to their preferred paw to prevent simultaneous use of the non-preferred paw [26]. Each rat received one daily test session consisting of 21 trials. The last 5 sessions were used in calculating average baseline performance. A reach was considered a "success" if the rat grasped the pellet, brought it inside the box using its paw, and placed the pellet into its mouth. The reach was labeled a "hit" when at least two reaching attempts were made in order to retrieve a pellet. A "failed" reach is one in which an animal advanced the paw through the slot but missed the pellet or knocked it off the ledge. Reaching success (for each type of reach) was defined as [number of successful retrievals / 21] × 100.

The last training day was videotaped (Sony digital video camera recorder; model DCR-HC21; shutter speed of 1000th of a second) for subsequent kinematic analysis of reaching movements. The first three successful reaches were each analyzed qualitatively [16, 26]. We anticipated that only a small proportion of reaches would be successful after ICH, and therefore also analyzed movement components for reaches considered hits and

failures. Each reach was decomposed into 11 movement components: (1) orient to pellet, (2) limb lift, (3) digits close, (4) aim, (5) advance, (6) digits open, (7) pronation, (8) grasp, (9) supination I, (10) supination II, and (11) release. These movements were further divided into a total of 35 subcategories, and each of these was rated as 0 (loss of normal movement), 0.5 (impaired but recognizable movement pattern), or 1 (normal movement). A score of 35 denotes a perfect reach.

7-2c Intracerebral Hemorrhage Surgery

Rats were anesthetized with isoflurane (4% induction, 2% maintenance in 70% N_2O and 30% O_2) and placed in a stereotaxic frame. Using aseptic technique, a midline scalp incision was made and a hole was drilled 3.5 mm lateral to Bregma, contralateral to the preferred paw as determined in the single pellet task [16]. To create an ICH, a needle was inserted 6.5 mm below the surface of the skull and 100 μL of blood, taken from the tail vein, was infused into the lateral striatum over 10 minutes [18, 20]. The needle remained in place for an additional 10 minutes to prevent blood from backing up the needle tract. A metal screw (Model MX-080-2; Small Parts, Miami Lakes, FL) was inserted into the thickness of the bone and the wound closed with staples. A local anesthetic (Marcaine; Sanofi Canada, Markham, Ontario, Canada) and antibiotic ointment were applied to the wound at the end of surgery. Rectal temperature was maintained at normothermia (~ 37 °C) during surgery (~ 45 min) using an electric heating blanket.

7-2d Skilled Reaching Testing

Three days prior to each test session, rats were food deprived to 90% of free feeding weight. On days 7 - 11 and 28 - 32 following ICH, rats were tested once daily in both the staircase and single pellet reaching tasks. The order of testing was alternated

daily and sessions were separated by at least 4 hours. In the staircase task, we videotaped performance on days 11 and 32 post-ICH and counted the number of reaches made with each forelimb [29]. A reach occurred when the rat advanced the paw from the central platform to the stairs and grasped at a pellet. We calculated the number of reaches made per pellet retrieved in each 15 – minute test session. In the single pellet task, performance was videotaped on days 11 and 32 for kinematic analysis of reaches.

7-2e Histology

Rats were euthanized 33 days following ICH with an overdose of sodium pentobarbital (85 mg/kg; i.p.), and were transcardially perfused with 0.9% saline followed by 10% formalin. Forty μm coronal sections were taken every 400 μm using a cryostat and stained with cresyl violet. Using Scion Image J (v. 4.0), lesion volume was calculated as routinely done [30, 31]:

Volume of tissue lost = Remaining volume of normal hemisphere – remaining volume of injured hemisphere.

Volume of a hemisphere = (area of remaining tissue × distance between sections × number of sections analyzed).

7-2f Statistical Analysis

Using SPSS (v. 14.0), reaching success was assessed using repeated-measures analysis of variance (ANOVA; 2 within-subjects factors). Mann-Whiney U tests were used for ordinal data (i.e., rating scale for qualitative analysis of reaching). We performed linear correlation analyses (Pearson r) to determine whether skilled reaching performance (% reaching success) related to histology. Data are expressed as mean ± standard error of the mean, or as median scores.

7-3 Results

7-3a Staircase Task

One rat was excluded from this task because of failure to meet criterion during training and was thus excluded from analysis. Contralateral forelimb reaching success was significantly impaired on days 7 - 11 after ICH (40.17 % success ± 6.07 vs. 65.28 % ± 6.07 at baseline; $p = 0.006$). Rats retrieved more pellets on subsequent days during this first test session ($p = 0.031$). In the second test session (days 28 - 32 post-ICH), reaching success significantly improved (to 50.65 % success ± 5.71; $p = 0.002$ vs. first test session) and was not significantly different from baseline performance ($p = 0.059$; Fig. 2A). Ipsilateral forelimb reaching success was not impaired at either time point (Fig. 2B). Furthermore, ipsilateral reaching success in the second test session was significantly greater than the first session ($p = 0.001$) and baseline performance ($p = 0.008$).

With the contralateral forelimb, rats made an average of 17.99 ± 4.43 reaching attempts for each pellet retrieved on day 11. This was significantly reduced to 7.00 ± 1.63 reaches / pellet on day 32 ($p = 0.018$). Success rate with the ipsilateral forelimb also improved between days 11 and 32 (from 8.01 ± 0.88 reaches to 4.42 ± 0.58 reaches / pellet; $p = 0.001$).

7-3b Single Pellet Task

Reaching Success

Rats successfully retrieved a pellet on 60.43 % ± 3.12 of reaches prior to ICH (last 5 training trials). One rat switched limb preference after ICH (e.g., reached with initially non-preferred paw) and was excluded from analyses. Reaching success was severely impaired in the first test block (days 7 – 11) after ICH (16.97 % success ± 4.90;

$p < 0.001$ vs. baseline). Modest but significant improvement was detected by the second test block (to 30.13 % success ± 5.78; $p < 0.001$ vs. first test block). However, rats were still significantly impaired relative to baseline ($p < 0.001$; Fig. 3).

Reaching Movement Components

In Figure 4, the mean ranked scores for movement components (orient, limb lift, digits close, aim, advance, digits open, pronation, grasp, supination I, supination II, and release) are shown for successful reaches at baseline and days 11 and 32 post-ICH. Movement scores indicate that that pronation ($p = 0.033$), grasping ($p < 0.001$), supination I ($p = 0.028$), supination II ($p < 0.001$), and pellet retrieval ($p = 0.050$) were impaired 11 days after ICH. By day 32, however, only digit opening ($p = 0.047$) and supination II ($p = 0.003$) remained impaired. Prior to ICH, rats advance the paw through the slot and pronate the paw over the pellet in an arpeggio movement (Fig. 5A). The digits close around the pellet (Fig. 5B), and the paw is supinated such that the palm is oriented vertically to withdraw it through the slot (Fig. 5C). The paw is further supinated so the palm faces the mouth, the digits open, and the pellet is placed into the mouth. The head and upper body are lowered and the non-reaching paw is lifted to support the preferred paw (Fig. 5D). Rats display abnormal movements when retrieving the pellet after ICH. The paw is often fully pronated and moves either laterally (from the side) over the pellet, or the rat slaps at the pellet (from above; Fig. 5E). The ICH rats are often unable to properly close the digits around the pellet and drag the pellet to the slot without lifting the paw (Fig. 5F). Rats also fail to supinate the paw completely and place the snout into the slot to retrieve the pellet with the teeth (Fig. 5G). When the paw is withdrawn through the slot, ICH rats frequently rotate the body and "chase" the pellet with the snout

instead of opening the digits and placing the pellet into the mouth (Fig. 5H). The non-reaching limb is seldom raised for support when retrieving the pellet.

Rats often retrieved the pellet using multiple reaches (a "hit"). Eleven days after ICH, aim ($p = 0.030$), grasping ($p = 0.020$), and supination II ($p = 0.001$) were impaired in these reaches. By day 32, only supination II was impaired ($p = 0.004$). Movement components during these reaches were similar to those described above. Failed reaches typically occurred when the fully pronated paw slapped at the pellet and knocked it from the ledge. For failed reaches, abnormal movements were detected for opening the digits ($p = 0.001$) and for pronation ($p = 0.005$) on day 11, and for pronation on day 32 ($p = 0.014$). Movements for grasping, supination, and release were not analyzed because they typically did not occur in failed reaches.

Reaching Movement Rating Score

In Figure 6, the movement rating scores for successful reaches, hits, and failures are shown for baseline and on days 11 and 32 after ICH. Frame-by-frame analysis of reaches revealed that movement scores for each type of reach were significantly decreased 11 ($p \leq 0.005$) and 32 days ($p \leq 0.043$) following ICH. For successful reaches, movement scores significantly increased between days 11 and 32 ($p = 0.003$).

7-3c Histopathology

Intrastriatal hemorrhage primarily damaged the striatum. Occasionally, other structures such as the globus pallidus and corpus callosum were injured (Fig. 7). The volume of tissue lost 33 days after ICH was 23.45 $mm^3 \pm 1.68$ and this value measures both lesion size (e.g., cavity and cellular debris such as degenerating erythrocytes) and atrophy (e.g., ventriculomegaly).

7-3d Relationship Between Reaching Success And Histology

We did not find a significant correlation between lesion volume and reaching success in the staircase or single pellet tasks ($r \leq 0.391$; $p \geq 0.234$). This was likely due to the small degree of variability in lesion volume. As expected, however, there was a strong and significant relationship between performance in the staircase and single pellet tasks in the first ($r = 0.805$; $p = 0.005$) and second ($r = 0.690$; $p = 0.027$) testing sessions.

7-4 Discussion

This study shows that striatal infusion of autologous whole blood causes significant skilled reaching deficits in the staircase and single pellet reaching tasks. Although initially impaired in the staircase task, rats recovered to pre-surgical levels by one month. In contrast, skilled reaching remained markedly impaired in the single pellet task, though moderate recovery in reaching success and in reaching movements occurred. Our results indicate that the single pellet task is preferable to the staircase task, especially if long - term deficits are sought in this model.

In contrast to focal ischemia [32, 33] and collagenase - induced ICH, [16, 34] long-term deficits in the staircase task did not occur in the whole blood ICH model. Therefore, the staircase task is best suited to assessing injury within the first few weeks after whole blood – induced ICH. Several factors may explain the difference in reaching success in the staircase and single pellet tasks. For example, it may be easier to obtain the pellet in the staircase task because the rat can drag the pellet up the wall of the platform to the mouth instead of grasping the pellet and supinating the paw as described in the single pellet task. Furthermore, rats can make several reaching attempts to successfully retrieve a pellet in the staircase task, as opposed to only one reach in the single pellet task. In the

staircase task, skilled reaching recovers over one month, evidenced by the fact that they consumed more pellets and by a reduction in the number of reaching attempts per pellet obtained. These findings are consistent with recovery of skilled digit use after focal cortical injury in primates [35]. Presently, we did not assess whether improved reaching success was due to true recovery or compensatory movements because qualitative analysis of reaching movements has not been standardized for this task. However, reaching movements are similar in the staircase and single pellet tasks [29], and we therefore did not expect to gain additional information by performing such an analysis in this study.

The single pellet task is one of few behavioral tests sensitive to long - term deficits in the whole blood model of ICH, and thus we highly recommend it for this model. Despite the relatively small lesion size created in our study, limited recovery is similar to that after more substantial cortical [32, 36] and subcortical injury [16, 37]. Much greater improvements in reaching success occur after small to medium lesions of the motor [32, 38] and sensorimotor cortex, [32, 39] pyramidal tract [40, 41] and basal ganglia [32]. Kinematic analysis of reaching movement components in our study revealed impairments in grasping and pellet retrieval, which greatly recovered over one month. The type of movement deficits and recovery detected here differs from those after subcortical ischemic [37] and hemorrhagic injury [16], in which deficits in grasping persisted. This is likely due to the greater extent of injury in the latter studies, which could include damage to other regions of the basal ganglia responsible for grasping movements. Our findings add to our previous work in ICH [16, 20, 30, 31] and cumulatively suggest that in many behavioural tasks deficits in the whole blood model are much less severe than occur in

the collagenase model. Furthermore, there is a greater degree of spontaneous recovery in the whole blood model. These behavioural differences may be explained by variations in ICH lesion location, severity, and pathophysiology (e.g., inflammation). For instance, lesions created by blood infusion are smaller than those in the collagenase model because blood dissects along the needle tract and white matter (e.g., corpus callosum). Thus, the extent of grey and white matter involvement probably differs between the models. Furthermore, the collagenase model produces more damage to other structures, such as the internal capsule and thalamus, which contribute to functional deficits. Together, these results highlight the importance of identifying appropriate behavioral tests for each type of ICH insult used.

There are several limitations to this study. First, we assessed only skilled reaching deficits, and did not evaluate other tests sensitive to striatal injury (e.g., rotarod test [42], adhesive tape removal test [16]). A battery of tests is needed to characterize the broad range of deficits (e.g., walking, forelimb inhibition, neglect) that occur after ICH. Thus, further work must identify additional tests sensitive to this insult. Furthermore, we assessed whether these skilled reaching tasks were sensitive to ICH injury, and not whether we could detect effects of cytoprotection. Importantly, while many tests detect the presence of injury, they are somewhat insensitive to variations in lesion size [16]. Although we expect that these tasks would detect therapeutic effects in this model, this hypothesis must be tested. Third, we trained and tested rats in the staircase and single pellet tasks concurrently and repeatedly, thereby possibly enhancing spontaneous recovery. This potential confound (i.e., inadvertent rehabilitation), however, must be balanced with the need to track recovery and to comprehensively assess outcome (i.e., using multiple tasks).

Switching limb preference in the single pellet task is an additional concern and it occurred in one rat in our study. Substantial injury, as occurs in the collagenase model, would increase the frequency of this problem [16]. Many investigators circumvent this by using a bracelet (e.g., a adhesive tape wrapped around the wrist) to prevent the ipsilateral (i.e., initially non-dominant) limb from fitting through the slot [32]. Forcing rats to use their impaired limb in this manner is a form of constraint-induced movement therapy (CIMT), which improves outcome after ICH [43, 44]. We chose to avoid inducing CIMT in the present study in order to assess "spontaneous" recovery, and thus excluded this rat from analysis. However, using this technique may be necessary if more rats switch limb preference. Finally, there are a number of practical concerns with using these skilled reaching tasks in preclinical ICH studies. For instance, these tasks require weeks of training, food deprivation, and in some cases, detailed analysis of movements. Thus, cytoprotection or rehabilitation studies using large numbers of animals would be very time consuming, especially for the detailed analysis. We did not gain substantial information by analyzing "hits" and "failed" reaches in addition to the successful reaches. Thus, we do not recommend such additional analysis. Furthermore, reducing the number of testing sessions, or choosing to use one skilled reaching task may be a preferred solution.

Effective preclinical testing for prospective ICH therapies requires the use of sensitive behavioural tests. Our study shows that the single pellet task is well-suited to the whole blood model of ICH. This and our previous study [16] illustrate that the choice of behavioral tests depends on the model of stroke, the location and severity of insult, the timing of testing, as well as practical limitations [16]. For the whole blood model, we

recommend using the single pellet reaching task with other simple, less time-consuming tasks (e.g., forelimb placing score) known to be sensitive to long-term deficits in this insult [22]. We expect that such a battery will improve preclinical studies and help to identify truly effective therapies.

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Figure Captions:

Figure 7-1. The staircase (A) and single pellet (B) tasks were used to assess skilled reaching deficits after ICH in rats.

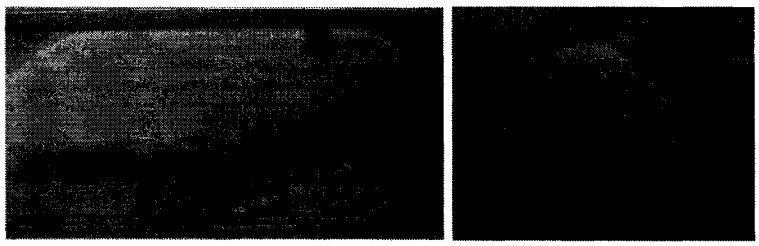


Figure 7-2. Reaching success with the contralateral (A) and ipsilateral (B) forelimb in the staircase test for the baseline period and on days 7-11 and 28-32 after ICH. Reaching was impaired the contralateral forelimb in the first test session. However, reaching success recovered to pre-surgical levels thereafter. The ipsilateral forelimb was not impaired after ICH.

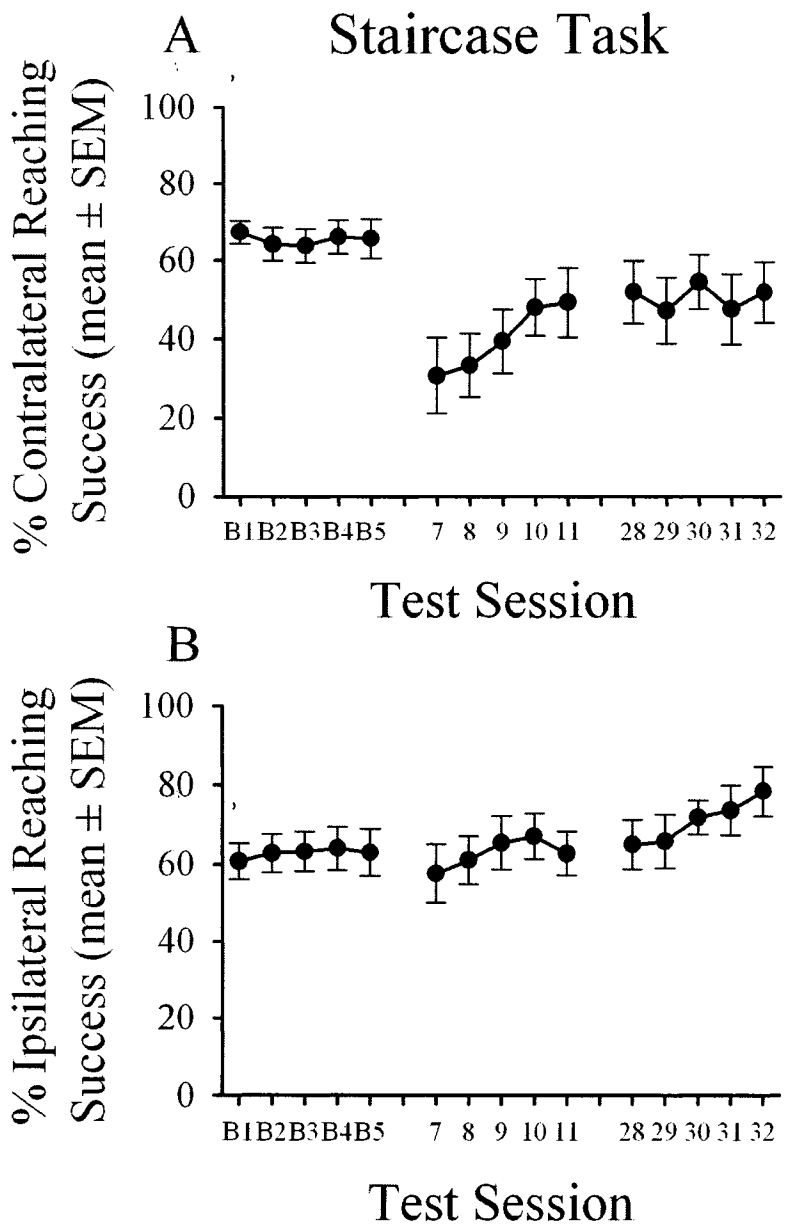


Figure 7-3. Reaching success in the single pellet task at baseline and on days 7-11 and 28-32 post-ICH. Skilled reaching was significantly impaired at both times. Modest but significant recovery occurred between test sessions.

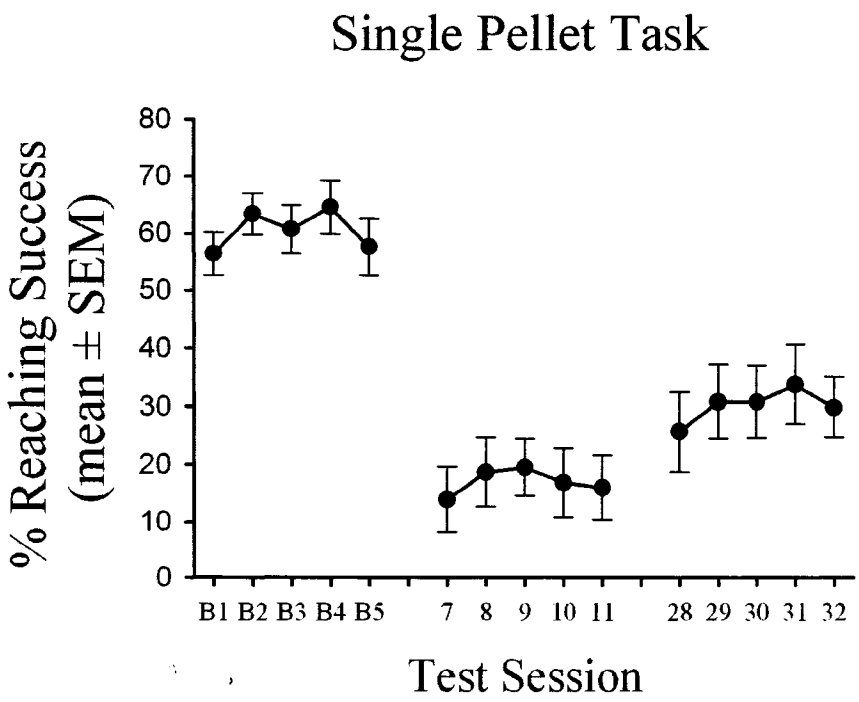


Figure 7-4. Mean ranked scores for the 11 reaching components at baseline and days 11 and 32 post-ICH for successful reaches (see Methods for description of movements). Results for "hits" and "failures" are described in the Results section.

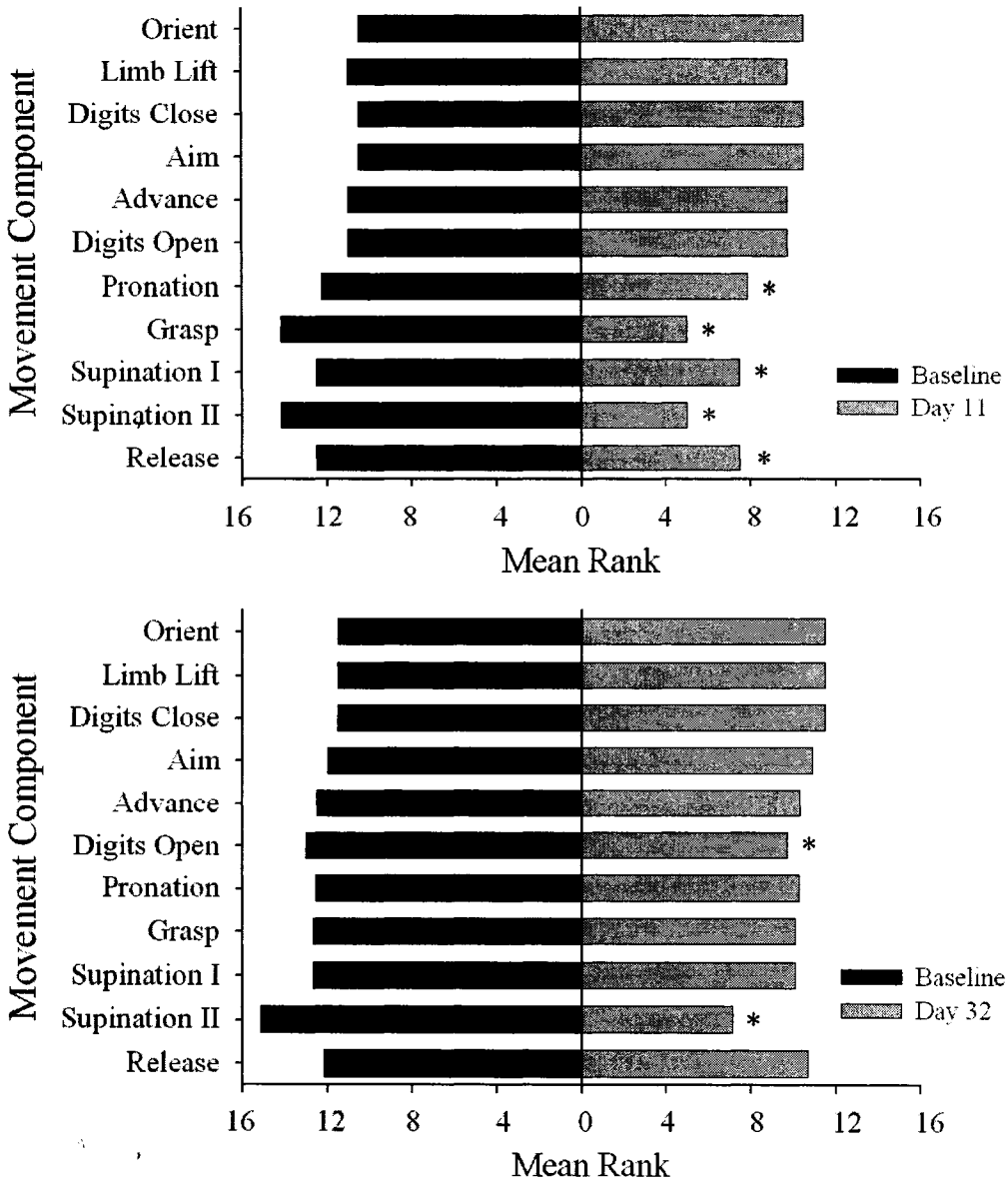


Figure 7-5. Photographs of a rat grasping for a food reward in the single pellet task. Normal movements for pronation, grasping, supination I, and supination II are shown in the top panel (A - D). Rats used abnormal reaching strategies 11 days after ICH (bottom panel; E - H). Supination remained impaired at 32 days post-ICH.

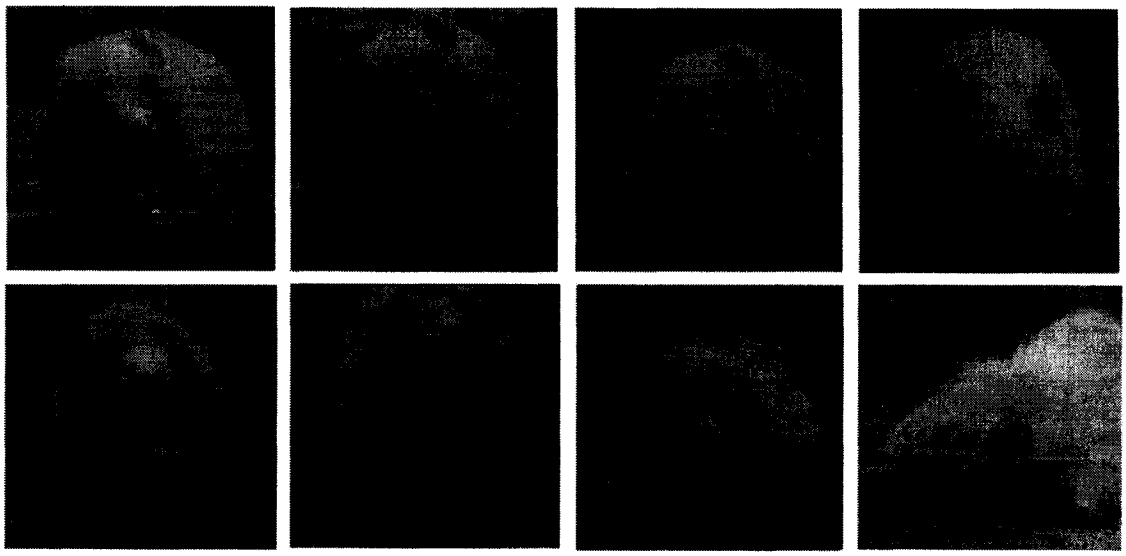


Figure 7-6. Median reaching movement rating scores for "success", "hits", and "failures" at baseline and on days 11 and 32 post-ICH. The overall scores on the 35-point scale were significantly reduced after ICH, although some recovery in successful reaches was detected by day 32.

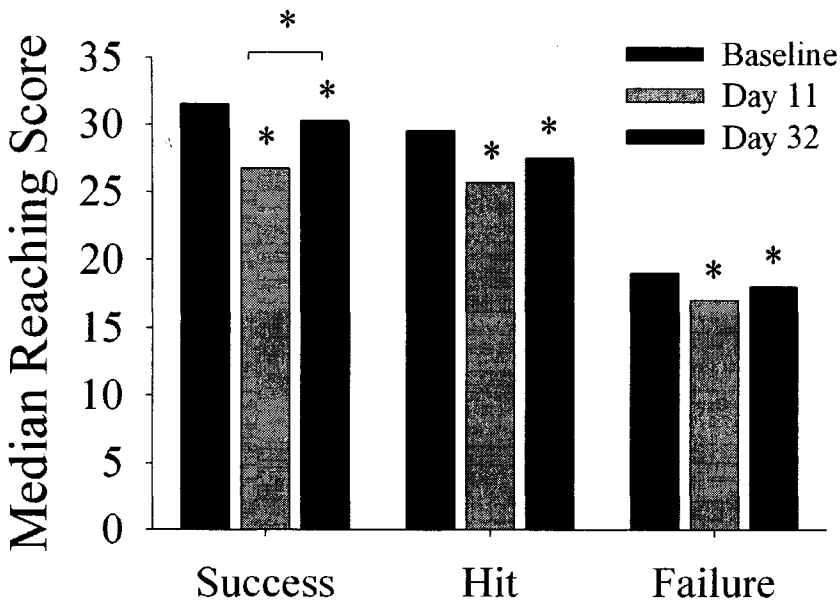
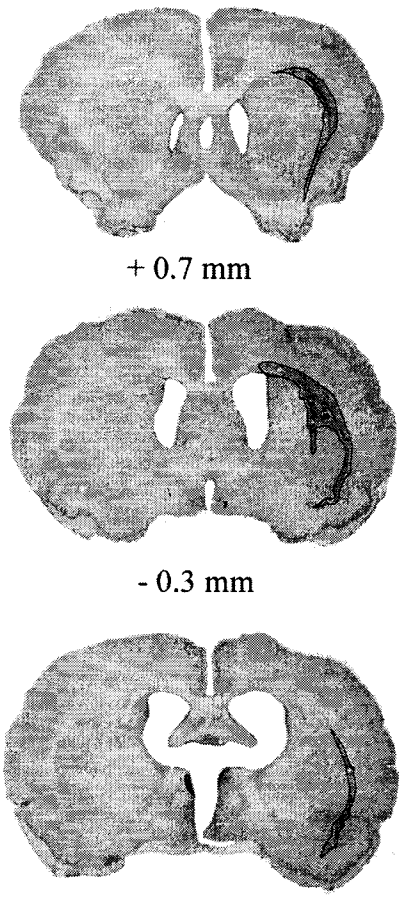


Figure 7-7. Photomicrographs of coronal sections represent the anterior, middle, and posterior sections of a typical lesion. Damage (outlined) occurred primarily to the striatum and white matter tracts, and extended from approximately +0.7 mm to -1.3 mm to Bregma. The volume of tissue lost at 33 days post-ICH included lesion (e.g., cavity, erythrocytes and cellular debris) and atrophy (e.g., ventriculomegaly), and was 24.35 $mm^3 \pm 1.68$.



+ 0.7 mm

- 0.3 mm

- 1.3 mm

Chapter 8: General Discussion

8-1 Introduction

The focus of this thesis was to evaluate the efficacy of mild and prolonged hypothermia in two rodent models of ICH. We obtained long-term histological and functional protection in the collagenase model when hypothermia was delayed for 12 h after ICH[1]. In the blood infusion model, early hypothermia reduced secondary consequences of ICH, including edema, inflammation, and BBB disruption, but this did not translate into long-term benefit[2]. Together, these data provide substantial evidence that hypothermia is a promising treatment for ICH. However, before ICH patients are treated with hypothermia, it must be further improved. Additional work is needed to identify the ideal hypothermia treatment and to limit or avoid side effects such as coagulopathy.

Another goal of this thesis was to refine functional assessment in the two models of ICH. Functional recovery is the endpoint of greatest clinical concern and therefore should be rigorously evaluated in cytoprotection studies[3-6]. For the collagenase model, we identified a battery of behavioural tests sensitive to a range of deficits after ICH and to gradations in injury akin to cytoprotection[7]. Long-term assessment in the whole blood model is problematic because rats show almost complete spontaneous recovery on most tests[2]. However, we found that skilled reaching is persistently impaired in this model, and that tests of skilled reaching can be used to assess long-term functional deficits[8]. Hopefully, thorough assessment of functional outcome will improve the quality of preclinical ICH studies and increase the likelihood of identifying a truly effective treatment for ICH.

8-2 Hypothermia

In Chapter 2, we tested whether prolonged and mild hypothermia would improve outcome after ICH induced by striatal infusion of bacterial collagenase. Our hypothermia treatment, which began 1 h after ICH, provides remarkable protection in rodent models of global[9, 10] and focal ischemia[11, 12]. Surprisingly, this treatment did not reduce brain injury or functional deficits after ICH[13]. We suspected that deleterious side effects of the early phase of hypothermia (i.e., soon after the ICH), such as elevated BP or coagulopathy, negated the beneficial effects of prolonged treatment and resulted in no net benefit. Therefore, we tested the efficacy of delayed hypothermia treatments (Chapter 3) and found that hypothermia was most effective when delayed for 12 h after ICH[1]. In accordance with our previous study, we found that earlier treatments (e.g., 1 or 6 h delay) did not improve outcome. Furthermore, a brief period of cooling induced soon after ICH exacerbated bleeding, in part by elevating BP. In the autologous blood infusion model of ICH, protracted bleeding does not occur[14]. Thus, we expected that early hypothermia would not exacerbate bleeding and would improve outcome in this model (Chapter 4). Although hypothermia reduced components of injury such as edema, inflammation, and BBB disruption, this did not translate into long-term histological or functional benefit[2].

Although promising, these data suggest that hypothermia provides substantially less benefit after ICH than after global and focal ischemia. There are several possible explanations as to why this occurs. First, it appears that the striatum is a difficult structure to protect. In fact, several studies of hypothermia after focal ischemia found better protection in the cortex than the striatum[12, 15-17]. This may be due to vascular differences or intrinsic differences in cell types (e.g., striatum vs. cortex or CA1 cells of the

hippocampus). Thus, it is possible that hemorrhagic insults in other structures such as the cortex or cerebellum are more amenable to hypothermia therapy, and this should be assessed. A better understanding of the striatum might help explain this discrepancy. Second, hypothermia is a potent anti-ischemia agent, but the presence of an ischemic area of tissue surrounding the hematoma has not been confirmed[18]. Therefore, mechanisms thought to contribute to ischemic injury may not *substantially* affect hemorrhagic insults. For example, anti-ischemic agents reduce behavioural deficits, but do not affect the amount of brain injury after ICH[19]. Other anti-ischemic agents provide no long-term functional or histological benefit[20]. However, we expect that hypothermia effectively treats life-threatening edema and elevations in intracranial pressure.

Another consideration is that we did not determine the optimal hypothermia treatment for ICH. In global and focal ischemia, hypothermia provides greatest benefit when it is initiated as soon as possible after the insult[11, 21, 22], and when cooling is protracted (e.g., 48 h)[9, 10]. Interestingly, our finding that hypothermia is most effective when delayed for 12 h after collagenase-induced ICH is in stark contrast to findings in ischemia. However, this may not be the case in the whole blood model, where 1 h delayed cooling sometimes provided greater benefit (e.g., significantly reduced edema) than the 4 h delayed treatment. The ideal hypothermia treatment, such as starting time or duration of cooling, may critically depend on the type of insult as well as the model used. It is possible that longer periods of cooling provide greatest benefit for ICH. Because degenerative events (e.g., inflammation, edema, oxidative damage) occur for several days following ICH[23, 24], it is reasonable to expect that a more protracted period of cooling (e.g., 4-5 days) would be needed to effectively attenuate these events. Several studies

found that hypothermia does not improve outcome for patients with traumatic brain injury (TBI)[25-27]. However, a recent study showed that a five-day period of cooling was needed to obtain benefit for TBI whereas shorter periods (e.g., 2 days) were ineffective[28]. Because TBI and ICH share similar mechanisms of injury, such as bleeding, edema, elevated ICP, and neurotoxicity of blood components, one could hypothesize that more protracted cooling would be most effective for ICH as well. We have no direct evidence and do not believe that shorter bouts of hypothermia (e.g., 24 h) are effective for ICH. Pilot data in the blood infusion model suggest that cooling to 33 °C for 24 h after ICH provides no long - term benefit (unpublished data). In order to optimize hypothermia treatment, future studies should examine factors that will critically affect ICH outcome, such as the optimal depth and duration of cooling and delay to initiation.

Our method of inducing hypothermia causes adverse systemic side effects that may limit its use and perhaps its efficacy. For example, cooling conscious rodents with fine water misters and fans is likely stressful and leads to elevated BP that, at least in part, exacerbates bleeding after ICH[1]. Other side effects include shivering, cardiovascular complications, and increased risk of infection[29, 30], which may counter the beneficial effects of hypothermia. Thus, an alternate method of cooling that does not produce such side effects would likely afford a safer and more effective hypothermia treatment. Anesthetizing animals for prolonged periods leads to excessive mortality; therefore exposure techniques are more appropriate for inducing prolonged mild hypothermia[31]. A method for inducing brain hypothermia in awake animals was tested in our laboratory[32]. This method involves placing a cooling coil that is flushed with cold water under the temporalis muscle and on the surface of the skull. A preliminary study showed that this

method reduced ipsilateral striatal temperature to ~33 °C for 24 hours in conscious rats. Importantly, cooling did not alter heart rate and BP. Additional studies will assess whether greater reductions in brain temperature can be achieved for prolonged periods (e.g., several days) and whether brain hypothermia effectively treats ICH in rats. Others investigated a similar approach and placed the "ChillerPad System" (also flushed with cold saline) onto the dura to induce focal brain cooling[33]. Following ICH in pigs, profound and prolonged brain hypothermia (surface temperature of 14 °C for 12 h, initiated 3 h after ICH) reduced vasogenic edema and IL-1β RNA levels, which are linked to BBB disruption. While these results are promising, it should be noted that hypothermia treatment, regardless of the method of induction, can induce coagulopathy by kinetic inhibition of clotting factors and prolong bleeding in ICH. Thus, limitations such as these must be identified and countered (e.g., with hemostatic therapy) before hypothermia is safely and effectively induced in ICH patients.

Several other factors influence the efficacy of hypothermia. Notably, insult severity dramatically affects the degree of neuroprotection in ischemia. Hypothermia provided near perfect protection following a 3 minute global ischemic insult in gerbils, but very little protection against a 5 minute insult[9]. Similarly, hypothermia reduced injury in temporary but not permanent focal ischemia[34]. It is possible that injury occurs very quickly and is so severe following a large ICH that treatments efficacious in moderate insults may fail to provide benefit. This appears to be the case in our recent study[35]. Delayed hypothermia treatment provided long-term histological and functional benefit after a moderate-sized ICH[1], but failed to improve outcome after a very severe insult[35]. Alternatively, following a very mild hemorrhagic insult, secondary consequences of

injury (e.g., elevations in edema) may be small and may not *substantially* contribute to injury. Hypothermia might be less efficacious for mild hemorrhagic insults if attenuating these processes does not lead to improved long-term outcome. This may explain why hypothermia did not provide long-term histological or functional benefit in the whole blood ICH model even though pathological processes (e.g., edema, inflammation, BBB disruption) were attenuated[2]. Accordingly, hypothermia might be most efficacious for "middle-of the road" insults, but we did not directly test this hypothesis.

8-3 Hyperthermia

Elevated body temperature on admission or in the days after ischemia increases morbidity and mortality[36-38]. Similarly, hyperthermia increases brain injury, functional deficits, and mortality in rodent models of global[39-41] and focal ischemia[42-45]. Hyperthermia also increases hemoglobin concentration in the brain following TBI in rats[46]. However, despite the fact that increased body temperature commonly occurs in ICH patients[47], few studies have examined the effects of hyperthermia on ICH outcome. Based on the clear relationship between fever and worsened outcome in ischemic stroke, we expected that hyperthermia would exacerbate injury and functional deficits after ICH. Unexpectedly, mild to moderate hyperthermia did not affect bleeding or inflammatory cell infiltration, and had no effect on short-term (7 days) or long-term (30 days) histological or functional outcome[48] (Chapter 5).

These curious findings are in sharp contrast to the ischemia literature, and this is likely due to fundamental differences in the pathology of ischemic and hemorrhagic insults. Hemorrhagic injury occurs rapidly and is caused by direct tissue destruction and space-occupying effects of the hematoma[49]. Thus, mechanisms thought to exacerbate

ischemic injury may not substantially contribute to ICH injury (e.g., bleeding, mechanical tissue destruction, neurotoxicity). In support of this idea, a similar hyperthermia regimen to the one used in our study increased brain injury, functional deficits, and mortality in rodent focal ischemia[42]. Perhaps ICH injury would be exacerbated if body temperature was elevated to a greater degree. Alternatively, the severe ICH produced in this study may not have been amenable to manipulation. Instead, hyperthermia may exacerbate mild ICH insults, and this should be assessed in the collagenase and the blood infusion models. The current findings are intriguing given that hypothermia improves outcome after ICH[1], indicating that ICH is indeed amenable to temperature manipulation. Differences between the studies are probably due to variations in experimental design, such as the degree of temperature manipulation (e.g., 4 °C reduction vs. 1.5 °C increase) or the duration of treatment. Regardless, these findings support our hypothesis that ICH is not as sensitive to temperature changes as ischemia. Nonetheless, until we have a better understanding of how hyperthermia affects ICH outcome, we suggest that hyperthermia be avoided for all stroke patients.

8-4 Assessment of Functional Outcome

Of a number of recommendations made to improve the quality of preclinical stroke studies[4, 5, 50], one that stands out is that the evaluation of infarct volume as a measure of outcome is no longer adequate. Instead, clinical trials should be based on preclinical evidence demonstrating improved functional outcome at long survival times measured on standardized batteries of behavioural tests validated for the model. Although a similar recommendation has not been formally made for preclinical ICH studies, we feel that rigorous assessment of functional outcome is also essential for identifying

effective therapies for ICH. Thus, a focus of this thesis was to characterize behavioural deficits and recovery in the collagenase and blood infusion models of ICH, and to refine functional assessment in these models (Chapters 6 and 7).

Our work has demonstrated profound differences in the type and severity of deficits, and the rate of spontaneous recovery in these ICH models. For instance, rats are severely impaired on a broad range of tests (i.e., of skilled reaching, walking, neglect, and NDS) in the first few days after collagenase-induced ICH and slowly recover thereafter[7]. Subtle impairments on many of these tests subsist over one month, even following mild insults. In contrast, by one week following striatal blood infusion, rats exhibit either slight or no deficits on tests shown to be sensitive to collagenase-induced ICH (e.g., ladder walking test, cylinder task, NDS) [2], (unpublished data). Assessing long-term outcome is therefore problematic in the whole blood model. In fact, we detected persistent deficits (e.g., at one month) only in the single pellet skilled reaching task[8]. The differences between the models may be explained by variations in ICH lesion location, severity, and pathophysiology. For example, lesions created by blood infusion are smaller than those in the collagenase model because blood dissects tissue along the needle tract and white matter (e.g., corpus callosum). Furthermore, our recent work using MRI to compare the evolution of ICH in the blood infusion and collagenase models suggests that the hematoma resolves faster in the whole blood model[14]. Thus, it is reasonable to expect that concomitant functional deficits resolve faster as well. Finally, the collagenase model produces more damage to other structures, which could contribute to functional deficits. These findings highlight the importance of identifying appropriate behavioural tests for *each* model of ICH.

We have also shown that a battery of tests should be used to assess the broad range of deficits that occurs following ICH. Using the collagenase model, it was clear that groups with different ICH volumes were easily distinguished by a battery of tests more so than with any particular test[7]. Thus, multiple tests should be used in cytoprotection studies, and each test should be sensitive to the ICH as well as to cytoprotective effects. Otherwise, treatment effects may be missed, especially if they are small. Perhaps this occurred in our study of the efficacy of hypothermia in the whole blood ICH model (Chapter 4). Because rats spontaneously recovered on each of the tests we used to assess function, it is possible that we simply missed a treatment effect. The addition of more sensitive tests for the whole blood model, such as the single pellet task, would have provided us with stronger evidence as to whether or not hypothermia afforded long-term functional benefit. Further testing could identify deficits in other behaviours such as grooming or play that may not be expected.

There are a number of issues to consider when selecting behavioural tests for ICH studies. First, behavioural deficits depend on the location of injury, severity of the insult, and timing of testing[7]. In addition, the choice of tests should take into account practical concerns such as the time needed to conduct testing or detailed analysis, and whether food deprivation is needed (e.g., in skilled reaching tasks). For practical and economic reasons, it is often hard to justify experiments designed to measure functional outcome at three months in a large number of animals, as required for Phase III trials in patients[51]. However, it is essential to rigorously evaluate functional outcome at long survival times at some point in preclinical testing. We recommend that researchers use a battery of behavioural tests that are sensitive to a broad range of deficits for their particular model

of ICH. For example, a skilled reaching task could be used in conjunction with other less time-consuming tests such as the NDS, adhesive tape removal task, or forelimb- placing test.

8-5 Limitations

Several basic concerns plague stroke studies to the point where one must doubt the value of using cytoprotective agents to treat stroke in humans. For example, is cytoprotection a reasonable strategy and will it ever play a clinical role? While promising results of cytoprotectants have been found in animal stroke models, all Phase III trials conducted to date have failed to provide benefit for humans[5]. Second, do animal models have predictive value for human stroke[52]? Is it possible to translate from the animal model to the clinical situation? Because of these questions, scientists have reevaluated the evidence to support cytoprotection in both experimental and clinical studies. It appears that the disappointing results of cytoprotection trials might largely be due to methodological problems[4, 5, 53].

Research achievements for ICH have been modest, in part because of limitations of animal models to reproduce the clinical situation[53]. Several species have been used to model cerebral hemorrhage, including rat, rabbit, cat, dog, pigs and primates[54, 55]. In rodents, the whole blood and collagenase models are commonly used, but neither model accurately reflects human ICH. For example, the whole blood model produces little injury and subtle functional deficits that recover spontaneously[2]. Furthermore, it does not mimic the ongoing bleeding that occurs in ~ 30% of ICH patients[56, 57]. The collagenase model produces more substantial injury and deficits[1, 7, 35, 48], but the widespread degradation of cerebral blood vessels and potential toxicity of collagenase limit its

clinical relevance[53]. Generally, the evolution of injury differs between rodents and humans. First, it appears that the breakdown of the hematoma[14] and progression of pathological processes such as edema and inflammation[24] occurs much faster in rats. Rodent ICH studies also focus mainly on gray matter injury, whereas models that involve both gray and white matter injury are desperately needed[53]. This requires using species with larger quantities of white matter such as swine or primates. At some point in preclinical testing, cytoprotectants should be tested in larger, gyrencepahlic animals that better reflect the biology and mechanisms of human ICH.

A number of other recommendations have been made to address the limitations of experimental stroke studies[4, 5], and they can be applied to ICH. For instance, most studies do not assess the efficacy of cytoprotectants in young and aged animals of both sexes, and with comorbid conditions such as hypertension or diabetes. Physiological variables such as BP, blood gases, hemoglobin, glucose, and temperature should be monitored. In addition, studies should evaluate dose-response curves, toxicology, and the time window of opportunity for treatment. Finally, rigorous functional assessment involves using a battery of tests over at least one month, and histological analysis (e.g., volume of infarct) must then be determined.

Certainly, we could improve our own studies by addressing several of these suggestions. First, we used young male rats in all of the work in this thesis. However, we have also begun to examine cardiovascular effects of hypothermia in aged and spontaneously hypertensive rats[14]. Several studies showed that older age[58] and female hormones (e.g., 17β-estradiol)[59-61] affect outcome (e.g., NDS, edema) after ICH. Thus, efficacy of a cytoprotectant may critically depend on such factors. Second, we did not

systematically assess factors that would impact the efficacy of hypothermia, such as the optimal depth or duration of cooling, or the delay to treatment. Instead, we used the pattern of cooling shown to be most effective for ischemic injury. Our work demonstrates that this regimen is much less effective for ICH, and must be further improved. Third, although we did not directly assess how insult severity influences efficacy, we have some evidence that hypothermia is less effective against very severe insults[35]. Despite this, we expect hypothermia to be life saving in cases with excessive edema that could lead to brain herniation and death. Fourth, we identified several potentially deleterious side effects of hypothermia for ICH, notably the coagulopathy that could prolong bleeding and worsen outcome[1]. Such side effects may limit the use of hypothermia for ICH unless they are avoided or countered[29, 31]. Fifth, we assessed the effects of hypothermia on striatal ICH only, and not in other types such as cortical or cerebellar ICH, or subarachnoid hemorrhage (SAH). Finally, we did not assess cognitive impairments after ICH, and did not evaluate the effects of temperature changes on cognitive function in theses studies. We hypothesize that cognitive deficits present following ICH may affect both spontaneous and treatment-induced recovery. Unfortunately, performance on cognitive tests (e.g., Morris water maze) is confounded by concomitant motor deficits after ICH. Therefore, the development of cognitive tests that do not rely heavily on motor movements is greatly needed in preclinical stroke studies.

An additional limitation of all experimental ICH studies is that we do not know whether reducing secondary consequences of ICH (e.g., edema, inflammation) in rodents will translate into improved long-term benefit. In fact, functional improvement may occur without an apparent affect of the amount of brain injury[19, 35]. However, because these

processes likely contribute to ICH injury, it follows that reducing them will improve outcome. Many cytoprotection studies have found reductions in secondary consequences of ICH *without* long-term benefit. For example, we found that mild prolonged hypothermia reduced edema, BBB disruption, and infiltration of inflammatory cells following whole blood ICH, however this did not reduce brain injury or substantially improve functional outcome[2]. Whether a treatment reduces edema is often the only endpoint used in many studies, and whether reductions in edema persistently reduce functional deficits or the amount of tissue lost is often simply not assessed[62]. It is clear that excessive elevations in ICP and edema will contribute to mortality. However, when these processes are not life threatening, such as after a mild or moderate ICH insult, would decreasing them improve outcome? Finally, we do not know how other secondary consequences of ICH, such as MMPs or the complement system contribute to ICH injury, or if reducing them with a treatment such as hypothermia would provide long-term benefit.

We attempted to overcome the aforementioned limitations in several ways. For instance, we used relatively large sample sizes in all of our studies (~12 / group). We repeatedly assessed functional outcome using a battery of behavioural tests sensitive to ICH. In fact, we identified behavioural testing batteries appropriate for the collagenase and whole blood ICH models. In addition to histological outcome, we evaluated several other endpoint measures such as edema, inflammation, and BBB disruption. Furthermore, we attempted to refine the models of ICH used in these studies in order to reduce variability and increase statistical power. For example, an infusion pump with tubing attached to a cannula is routinely used to infuse blood into the striatum[63, 64]. However, we

found that blood adheres to the walls of the tube, thus reducing the amount infused into the brain. To overcome this limitation, we developed an alternative hand-injection method that does not involve tubing. Lastly, we identified some side effects of our hypothermia treatment, such as coagulopathy and elevated BP, which might limit its safety and efficacy. In summary, we attempted to improve the quality of our study design to rigorously assess the efficacy of hypothermia for ICH. Although this required large and labor intensive studies, such thorough assessment is essential for identifying and evaluating truly effective cytoprotectants.

8-6 Future Directions

8-6a Adjunct Therapies for Hypothermia

The use of hypothermia in conjunction with other therapies is an old idea that could benefit ICH patients. Combinations of cytoprotectants have been tested and are recommended[53, 65], and the combination of a cytoprotective agent and subsequent rehabilitative therapy should also provide benefit for ICH. Unfortunately, stroke rehabilitation studies often exclude ICH patients. However, recent clinical trials reported that ICH patients with severe levels of impairment benefit from rehabilitation[66]. In fact, although ICH patients had greater functional impairments than cerebral ischemic patients, they made greater gains[67]. Our recent study[35] was the first and only one to assess the combination of a cytoprotectant and rehabilitation for ICH. We tested whether the combination of delayed hypothermia followed by constraint-induced movement therapy (CIMT) would improve outcome after a severe ICH in rats. We created a large ICH in this study to test the hypothesis that the dual treatment is needed. Previous studies from our laboratory showed that each treatment on its own reduced brain injury and improved

functional outcome after a moderate-sized ICH[1, 68]. Although neither therapy alone provided much benefit for severe ICH, the combination of treatments reduced skilled reaching deficits. We suspect that hypothermia may have rescued some peri-hematoma neurons that CIMT then acted upon (i.e., neuronal remodeling), but the mechanisms of this interaction should be assessed in future studies. In addition, further improvements to hypothermia (e.g., optimized therapy or an alternate method of cooling) and / or to CIMT (e.g., less stressful method of limb restraint) might increase efficacy and make this combination useful for more severe insults.

Ultra-early hemostatic therapy for ICH has gained considerable interest and support over the past few years[69-72]. Imaging studies have provided evidence for the occurrence of early hematoma growth in approximately 30% of ICH patients[56, 73, 74]. This hematoma growth is likely due to rebleeding into damaged tissue surrounding the hematoma[75], and is associated with neurological deterioration. Recombinant activating factor VII (rFVIIa, NovoSeven®) inhibits fibrinolysis and activates coagulation locally, allowing fast and effective hemostasis without causing systemic adverse events[76]. A recent Phase IIB clinical trial tested whether rFVIIa would limit ongoing bleeding and effectively reduce hematoma growth when administered within 3 h of ICH onset[72]. The rFVIIa treated patients (160 μg/kg dose) had significantly reduced ICH volumes at 24 h, more favorable neurological outcome 3 months following ICH, and less mortality[72]. The results of this study are encouraging and a Phase III trial is currently underway. In addition, a recent rodent study found that rFVIIa reduces hematoma volume following collagenase-induced ICH, providing additional support for the use of rFVIIa for ongoing bleeding in ICH[77]. A further potential use for rFVIIa is in combination with hypothermia

after ICH. We hypothesize that rFVIIa would prevent the effects of hypothermic coagulopathy, and thus allow for earlier induction of hypothermia after ICH. We suspect that hypothermia will be most effective when induced as soon as possible after ICH, as is the case for cerebral ischemia. Thus, the combination of rFVIIa and hypothermia should effectively treat both primary ICH (e.g., hematoma expansion) and secondary injury (e.g., inflammation, edema, etc), resulting in better protection than with either treatment alone.

8-6b Alternate Methods of Cooling

Future studies should also examine the safety and efficacy of alternate methods of cooling. Several small clinical trials tested "cooling helmets" that aim to provide regional (brain) hypothermia and minimize systemic complications[78, 79]. A feasibility study demonstrated a brain temperature of < 34 °C achieved within ~ 4 h of cooling induction. However, it should also be noted that systemic hypothermia developed within ~ 7 h of cooling[78]. A small clinical study in ICH patients demonstrated that a local cooling technique reduced edema and functional impairments at 7 and 14 days after ICH[80]. However, Phase III trials have not evaluated the efficacy of this technique. Several laboratories have implemented a similar design for rodent studies. A surface cooling coil induced a 5 h period of moderate hypothermia following focal ischemia that reduced infarct volume without causing adverse physiological effects[81]. Several other promising studies evaluated local brain cooling, in which temperature was reduced in a region of the brain, as opposed to the entire brain or body. These studies demonstrated that mild to moderate hypothermia can be maintained in the underlying cortex and striatum for prolonged periods without cardiovascular complications[32, 33]. When applied to ICH in pigs, this local brain cooling technique reduced vasogenic edema and IL1-β levels[33]. The

efficacy of local brain hypothermia should also be assessed in rodent ICH using long-term histological and functional measures of outcome. Pharmacological means of cooling has also been tested. For instance, the efficacy of local cooling was studied for hemorrhagic stroke (ICH and SAH) using indomethacin (a non-selective cyclooxygenase inhibitor) and a modified nasopharyngeal cooling method[82]. It appears that 14 days of cooling induced in these patients prevented secondary brain damage (i.e., cooling reduced levels of IL-1β and bilirubin, as well as oxidative stress), though hematoma volume and neurological outcome were not assessed. Future studies should compare the safety and efficacy of brain cooling vs. whole body cooling. Such studies must determine whether the brain can be sufficiently cooled for prolonged periods, whether brain temperature can be precisely regulated, whether cooling the entire brain is more effective than cooling one region, and finally, whether adverse systemic complications eventually develop.

8-6c Role of Inflammation and Edema soon after ICH

One line of research that would be interesting to pursue is whether the inflammatory response and edema formation soon after ICH is *beneficial*. We generally assume that these processes are pathological and aggravate ICH injury. However, there is no conclusive evidence to support this idea. In fact, there is increasing evidence that some components of the inflammatory response (e.g., MMPs, the complement system, and microglia) have beneficial roles in ICH[24]. Components of the inflammatory response (e.g., neutrophils and macrophages) are also involved in degrading and removing erythrocytes and necrotic tissue[23]. Many blood-breakdown products such as hemoglobin and thrombin are neurotoxic[83-85], and may contribute to cell death following ICH. Thus, timely resolution of the hematoma could limit secondary brain injury. Furthermore, small

increases in edema soon after ICH may promote clotting via tamponade. Indeed, one clinical study showed that early edema formation is actually a predictor of *good* outcome in ICH patients[86].

If these processes contribute to clot formation and hematoma resolution, then it is reasonable to expect that reducing inflammation or edema in the *early* phase of ICH (i.e., during the first few hours) may not be beneficial, as previously assumed. Could inhibiting such processes actually impede recovery or contribute to worsened outcome? If this is the case, then early hypothermia treatment may not be indicated for ICH. We hypothesized that delayed hypothermia was most efficacious for ICH (Chapter 3) because earlier treatment aggravated bleeding. Thus, we expected to obtain superior benefit when hypothermia was induced soon after ICH in the whole blood model, in which continuous bleeding does not occur. However, although hypothermia reduced edema, BBB disruption and inflammation, we did not obtain long-term benefit. It is possible that inhibiting these processes soon after ICH was unfavorable and dramatically reduced the efficacy of our treatment. Future studies could assess these possibilities. A better understanding of the pathophysiology of ICH and of the beneficial or harmful roles of its components would certainly advance ICH research.

8-7 Summary

Despite the overwhelming amount of research being done to understand the mechanisms of ICH and identify effective therapies, a proven clinical treatment remains unavailable. However, the basis for cytoprotection for ICH, although plagued with numerous concerns, is well founded. The failure to translate positive findings from animal models to human ICH stems from methodological flaws in both experimental and

clinical trials. For example, preclinical assessment of cytoprotectants should include rigorous functional assessment in addition to histology, adequate sample sizes, and protracted survival times. In the experiments described in this thesis, we have attempted to address these considerations so that we may rigorously assess the therapeutic potential of hypothermia.

We tested the efficacy of mild and prolonged hypothermia in two rodent models of ICH. Hypothermia reduced brain injury and functional deficits in the bacterial collagenase ICH model, and decreased edema, BBB disruption, and inflammation in the whole blood model. These data provide strong evidence that hypothermia is a valuable cytoprotective agent for ICH, and its efficacy and mechanisms should be tested further. However, we suggest that hypothermia treatment can and must be further improved for ICH. For example, studies should identify and counter potentially deleterious side effects that may limit the safety and clinical efficacy of hypothermia. In addition, the optimal cooling regimen (e.g., depth and duration of cooling) and method of inducing hypothermia (e.g., systemic vs. local cooling) should be determined for ICH. The combination of hypothermia and other therapeutic interventions such as rFVIIa, rehabilitation, and other cytoprotectants may provide greatest benefit for ICH. Importantly, we believe that hypothermia should not be applied to ICH patients until such improvements are made. Nonetheless, we propose that hypothermia is a promising cytoprotective approach for hemorrhagic stroke and warrants further investigation.

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