Neuroprotective and Restorative Potential of Remote Ischemic Conditioning Following Stroke

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Thesis submitted to the University of Ottawa
in partial fulfillment of the requirements for
the Master of Science degree in Neuroscience

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ABSTRACT

Remote ischemic conditioning (RIC) is a noninvasive procedure where blood flow to a limb is repetitively reduced, sometimes called an "exercise memetic". RIC delivered before (pre-RIC) or after (post-RIC) stroke is reportedly neuroprotective in preclinical stroke models. A review of the preclinical RIC literature revealed that studies almost exclusively use male subjects and a single stroke model (MCAO) that produces a large injury (~34% of hemisphere). To improve clinical translation, efficacy should be demonstrated in multiple stroke models and both sexes. Furthermore, the restorative potential of RIC (delivered past the neuroprotection window) to improve stroke recovery remains to be investigated. In male and female Sprague-Dawley rats (n=129) a standardized session (5min inflation, 5min deflation, 4 repetitions) of RIC was delivered using a pressurized cuff on the hindlimb. RIC was either delivered once 18h before, once 4hr acutely after or daily for 28 days beginning day 5 after endothelin-1 (ET-1) stroke. Infarct volumes were assessed 24hrs after stroke using MRI. To determine if RIC efficacy varied across stroke size, a hierarchical cluster analysis was used to divide rats into subgroups based on stroke size (small/large). RIC was effective in ET-1 which produced smaller strokes ("small":5.2%, "large":18.0% of hemisphere) than MCAO (~34%). This is more comparable to injury sizes seen clinically (4.5-14.0%). "Small" ($42\pm4mm^3$) strokes were reduced by 39% ($p=0.010$, $d=0.29$) and "large" ($146\pm8mm^3$) strokes were reduced by and 35% ($p<.00001$, $d=1.41$). Pre-RIC reduced infarct volume by 41% ($p=<0.0001$, $d=0.92$) versus 29% ($p=0.009$, $d=0.43$) in post-RIC. Interestingly, RIC is more effective in males, with double the infarct volume reduction of 46% ($p<0.0001$, $d=0.94$) compared with 23% ($p=0.013$, $d=0.42$) in females. Although RIC did not show restorative potential to improve motor stroke recovery, RIC is

neuroprotective now with stronger clinically relevant evidence. RIC is effective across stroke models, stroke sizes and sex. Application of RIpreC to prevent stroke following a transient ischemic attack or recurrent stroke (especially in males with “large" strokes) would have the greatest potential.

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LIST OF ABBREVIATIONS

AIF	Apoptosis Inducible Factor
AKT	Protein Kinase B
ANOVA	Analysis of variance
AP	Anteroposterior
ATP	Adensoine Triphosphate
BCAO	Bilateral Carotid Artery Occlusion
BCAS	Bliateral Carotid Artery Stenosis
BDNF	Brain Derived Neurotrophic Grwoth Factor
C-RIpostC	Chronic-Remote Ischemic Post-conditioning
CBF	Cerebral Blood Flow
CCA	Common Carotis Artery
CREB	cAMP Response Element-Binding Protein
DV	Dorsoventral
ECA	External Carotid Artery
ET-1	Endothelin-1
EVT	Endovascular Thrombectomy
HIF	Hypoxia Inducible Factor
HSP	Heat Shock Protein
GABA	Gamma-Aminobutyric acid
GAP43	Growth Associated Protein 43
IC	Ischemic Conditioning

ICA	Internal Carotid Artery
IGF-1	Insulin—like Growth Factor-1
LDI	Laser Doppler Imaging
MCA	Middle Cerebral Artery
MCAO	Middle Cerebral Artery Occlusion
ML	Mediolateral
MRI	Magnetic Resonance Imaging
NO	Nitric Oxide
REGW-F	Ryan-Einot-Gabriel-Welch F
RIC	Remote Ischemic Conditioning
RIpreC	Remote Ischemic Pre-conditioning
RIperC	Remote Ischemic Per-conditioning
RIpostC	Remote Ischemic Post-conditioning
SD	Sprague-Dawley rat
SEM	Standard Error of Mean
STAIR	Stroke Therapy Academic Industry Roundtable
SRRR	Stroke Recovery and Rehabilitation Roundtable
TIA	Transient Ischemic Attack
TTC	2,3,5-Triphenyltetrazolium Chloride
VEGF	Vascular Endothelial Growth Factor

ACKNOWLEDGEMENTS

I would like to first thank my supervisor, Dr. Dale Corbett for giving me the flexibility to explore many facets of stroke. It is truly a privilege to be his final Masters student and to learn from someone so passionate and effective at what they do and love. Dr. Corbett was a true role model for ethics and living a healthy lifestyle both in and out of the lab.

I would also like that thank my co-supervisor Dr. Greg Silasi for taking me as his first Master's student. Despite the stress and workload of starting up a new lab he always made time to provide helpful input and review my work.

Thank you to members of my TAC committee, Dr. Diane Lagace and Dr. Simon Chen. You not only facilitated helpful critical discussions of my research but also provided encouragement and built my confidence throughout these past two years.

Thank you to Dr. Matt McDonald and Matthew Jeffers for daily discussions and providing ongoing advice. Matthew Jeffers statistical expertise was invaluable while Dr. McDonald's natural desire to teach and offer assistance was always welcome. Whether it be reviewing draft work or teaching lab techniques, he will make a great PI. Thank you for the undergraduate students Lydia Kuhl, Allyson Ripley and Lama Issa for always being eager to contribute and keeping things fun in the lab.

A special thank you to Ralph Nevins, Julian Pitney and Anthony Carter for quickly building a one-of-a-kind RIC apparatus that allowed to me to run multiple rats at a time. Anthony as well as Matthew Jeffers and Matthew McDonald also helped with a countless number of stroke

surgeries. Without everyone’s help, my project would not have been possible - especially within the short time frame!

Lastly, I would like to thank my family for supporting me throughout my continuous academic endeavors. Thanks for taking the time to listen when I was stressed, suggest solutions and motivate me when results and scheduling didn't always go as planned.

INTRODUCTION

1.1 Stroke: Epidemiology, Treatments, and Preclinical Models

With improved acute stroke care more people are surviving stroke, but are left living with chronic deficits that affect their quality of life. In fact, stroke is the leading cause of neurological disability in adults (Feigin et al., 2014; Ward, 2017). Disability rates among stroke survivors are on the rise in Canada with the current prevalence of 400,000 expected to double within 20 years (Krueger et al., 2015). Likewise, those who are more severely impaired (~50%) and depend on others for daily living (Heart and Stroke Foundation, 2017) would predictably also double, increasing the economic burden faced by households and the health care system (Mittmann et al., 2012). Deficits associated with stroke are not restricted to motor impairments, and symptoms such as post-stroke fatigue, deconditioning, and depression can have a negative impact on post-stroke recovery and rehabilitation (Eskes et al., 2015). Fatigue marked by weakness and exhaustion, is estimated to be experienced by up to 75% of people following stroke (Choi-Kwon & Kim, 2011). Post-stroke fatigue makes it more challenging for patients to adequately participate in intensive and effective rehabilitation for optimal recovery (Choi-Kwon & Kim, 2011; Eskes et al., 2015).

Strokes are either hemorrhagic or ischemic in nature. A hemorrhagic stroke occurs when a blood vessel ruptures and bleeds within the brain, while an ischemic stroke is caused from a blockage that prevents blood from reaching regions of the brain. Ischemic blockages caused by narrowing of a blood vessel from a clot are the most common cause of stroke, representing over 80% of strokes (Truelsen, Begg, & Mathers, 2006). This ischemia can cause both immediate and delayed cell death over days, resulting in permanently damaged tissue (infarct core). The peri-infarct

tissue surrounding this region undergoes considerable plasticity following stroke and represents potentially viable tissue which may be salvageable with therapeutic interventions (eg. re-perfusion or re-canalization) (Carmichael, 2016).

There are limited treatments for ischemic stroke. Tissue plasminogen activator (tPA or altepase) is the only approved drug which dissolves clots by producing plasmin to break down fibrin filaments. However, due to a limited time window (4.5 hours after stroke), patients are still often left untreated or tPA treatment is unsuccessful at breaking apart the clot (K. Lin et al., 2018). After this initial period, the risk of bleeding (intracranial hemorrhage) from tPA outweighs the benefits and endovascular thrombectomy (EVT) is the only other potential treatment. Surgical removal the clot by EVT can take place within the first 6 hours, or 24 hours for selective patients, based on Canadian Best Stroke Practice Recommendations (K. Lin et al., 2018). Although EVT has a larger treatment window, only about 10% (within 6 hours) (Vanacker et al., 2016) and 9% (within 6 to 24 hours) (Jadhav et al., 2018) of ischemic stroke patients are eligible. A worldwide ESCAPE trial of EVT treatment, including mostly Canadians, found that EVT resulted in successful re-canalization in 71% of patients (vs 31% non-EVT) and halved mortality to 10% (vs 19% non-EVT). However, these EVT effects are likely inflated as only highly efficient stroke centres with fast treatment times were included (Goyal et al., 2015). By 90 days post-stroke in patients from the ESCAPE trial, tPA administration alone or EVT were 37% (Hill & Buchan, 2005) and 53% (Goyal et al., 2015) effective at sending patients home without further rehabilitation. Despite these advancements in acute stroke care, chronic disability persists as treatments are limited in timing, efficacy and patient eligibility. To reduce long lasting impairments faced by stroke survivors, it is essential that novel strategies are developed to

reduce the impact of stroke and promote recovery beyond what can occur spontaneously (Ward, 2017).

Preclinical rodent models have played a substantial role in our understanding of post-stroke neuroplasticity and epochs of recovery (Murphy & Corbett, 2009). While mice are often used in preclinical research, the advantage of using rats is more extensive due to similar cerebrovasculature to humans in addition to larger brain structures that facilitate surgical manipulation. Rats also have more advanced learning and memory, that the capacity to assess post-stroke deficits with behavioural testing can be more fruitful (eg. cognitive deficits) (Iannaccone & Jacob, 2009). Although mice are often used for their transgenic strains (Kleinschnitz, Fluri, & Schuhmann, 2015), there is an increasing capacity for transgenic rat strains (Iannaccone and Galat 2014). In ischemic stroke, vessels can be *permanently* or *transiently* occluded with the latter permitting reperfusion after the occlusion (MacRae, 2011), similar to the action of successful tPA or EVT treatment.

There are two common *transient focal* ischemic stroke models used in rodents. The endovascular middle cerebral artery occlusion (MCAO) model is the most widely used (Howells et al., 2010). A coated filament is inserted in the external carotid artery (ECA) or common carotid artery (CCA) and advanced through the internal carotid artery (ICA) to occlude the beginning of the MCA (termed *proximal* MCAO) for 60 to 120 minutes before it is removed to allow reperfusion (Liu et al., 2009; Bacigaluppi, Comi, & Hermann, 2010; Carmichael, 2005). However, another approach also exists where occlusion occurs in a smaller vessel more distal from MCA (termed *distal* MCAO) (Bacigaluppi et al., 2010). Since the MCA and its associated branches account for the majority (70%) of human ischemic strokes (Bogousslavsky, Melle, & Regli, 1988), this model is representative of injury location in humans (Sommer 2008). However, with the MCAO

model mortality rate is high (only a 60-100% success rate) and injury size is very large (Liu et al., 2009). This is a significant limitation of this model, since lesions in the general stroke population are typically much smaller than those produced by MCAO (Edwardson et al., 2017). This disparity in injury size will likely have an impact on the translation of preclinical work using this model (Corbett et al. 2017). Further, due to the invasive nature of the procedure, complications can result in health issues that negatively affect research outcomes. For instance, the filament can occlude deep arteries not seen in humans leading to hyper- or hypo-thermia in rodents (Amki et al. 2015) or muscles involved in mastication can become damaged leading to feeding difficulties or post-operative weight loss (Sharkey & Butcher, 1995).

Alternatively, a less invasive method uses endothelin-1 (ET-1), a potent vasoconstrictor peptide. ET-1 can be injected cortically or into deeper brain regions to temporarily occlude cerebral vessels and induce focal ischemia (Sommer 2017). With stereotaxic injection, blood flow reduction by ET-1 can be targeted and localized to relevant areas such as sensorimotor, cognitive or striatal regions to produce more distinct functional deficits than the MCAO injury (Corbett, Jeffers, Nguemeni, Gomez-Smith, & Livingston-Thomas, 2015; Karthikeyan, Jeffers, Carter, & Corbett, 2019; Windle et al., 2006). In contrast to MCAO which exhibits rapid reperfusion (similar to EVT treatment which few patients receive), ET-1 induces gradual reperfusion (up to 48 hours), likely more representative of spontaneous reperfusion (Windle et al., 2006). As with any model, disadvantages of the ET-1 model also exist. For example, ET-1 receptors also exist in non-vascular cells (eg. neurons and astrocytes), which could potentially influence neuronal repair (Sozmen, Hinman, & Carmichael, 2012). *Focal* stroke can also be induced using photothrombosis, where after intravenous injection of a light sensitive dye (eg. Rose Bengal), light or laser exposure directed at targeted brain regions through the skull can cause clot

formation (Schmidt et al., 2012). Although this method is the least invasive and produces very localized injury, it results in *permanent* vascular occlusions to mainly cortical brain regions with more unconventional ischemic mechanisms (eg. severe edema, small penumbra) (Corbett et al. 2017), not representative of human strokes (Carmichael, 2005). Ultimately, this highlights the necessity of preclinical stroke interventions to be tested across multiple models (Corbett, et al. 2017).

1.2 Events Following Stroke: Cell Death, Neuroprotection, Heightened Plasticity and Spontaneous Biological Recovery

When investigating potential therapies to promote stroke recovery, researchers must understand the biological basis of events occurring after stroke. Within minutes of stroke neurons are deprived of oxygen and nutrients depleting their energy (Murphy & Corbett, 2009). During ischemia, neurons are unable to maintain proper ionic membrane balance, cascading into the release of cytokines activating proinflammatory immune cells and the release of excess glutamate causing excitotoxicity, leading to the generation of free radicals damaging cells through necrosis, apoptosis and autophagy. Neurons typically die by necrosis in the ischemic core where ATP is depleted and by apoptosis in the penumbra where sufficient energy levels remain (Majid, 2014). Neuroprotection strategies aim to reduce the amount of cell death of neurons, blood vessels or supporting glial cells in the penumbra before cell death is complete (24 hours in rats) (Tymianski 2013; Nguemeni et al. 2015). Interestingly, nuclear estrogen (estradiol) receptors are found throughout the brain (eg. neurons, astrocytes, microglia and endothelial cells) that may contribute to a degree of endogenous neuroprotection among females. For instance, estradiol has been found to increase dilation (via NO production from eNOS activation), reduce vascular inflammation (via interactions with claudin-5 at the blood-brain-barrier), enhance

mitochondrial function and decrease apoptosis (via inhibition of caspases) (Dubal & Wise, 2001; Engler-Chiurazzi, Brown, Povroznik, & Simpkins, 2017). In addition, female mice have been found to have elevated cerebral blood flow (CBF) and experience smaller stroke lesions than males (Alkayed et al., 1998). Restoration of blood flow is a major neuroprotective strategy. Since relatively few patients receive re-canalization by tPA or EVT due to risks of causing more damage (Neuhaus, Couch, Hadley, & Buchan, 2017) and spontaneous reperfusion is not guaranteed (Kassem-Moussa & Graffagnino, 2002) other neuroprotective approaches have been pursued. In efforts to reduce brain damage, many pharmacological agents have targeted specific pathophysiological pathways of stroke along the cell death cascade (eg. free radicals scavengers, ligands to reduce excitotoxicity, immune modulation etc.) (Rajah & Ding, 2017). Another approach is making the brain more resilient to ischemic injury with hypothermia and ischemic pre-conditioning affecting multiple molecular pathways (Tymianski, 2013; Rajah & Ding, 2017). Despite the promising potential of neuroprotection studies in preclinical models of stroke to date, none have shown clinical benefit (Patel & McMullen, 2017). To improve the translational potential of these neuroprotective approaches, there are now international research guidelines from the Stroke Therapy Academic Industry Roundtable (STAIR) whereas the Stroke Recovery and Rehabilitation Roundtable (SRRR) provides unique guidelines for recovery research. Both Roundtable organizations recommend that preclinical research demonstrate replication across different stroke models, replication within and between labs, use different species and both sexes, and incorporate age and disease comorbidities into existing models (Corbett et al. 2017; Stroke Therapy Academic Industry Roundtable (STAIR) 1999).

Ischemic stroke causes permanent brain damage with early cell death, it also elicits an environment of heightened neural plasticity for a critical time period, similar to a developing

brain (Corbett et al., 2015; Corbett et al., 2017) when recovery promoting interventions are most effective. The neural balance of excitatory glutamate and inhibitory GABA signalling altered after stroke allows the brain to respond to external experiences. Upregulation of neuronal growth-promoting genes also supports neuroplasticity until growth -inhibitory genes predominate towards the end of the critical period (Carmichael et al., 2005) (**Figure 1**). This functional plasticity likely drives the observed structural regenerative processes such as axonal sprouting, neurogenesis (Jin et al., 2006) and gliogenesis (Sanin, Heeß, Kretzschmar, & Schüller, 2013) necessary for remodeling and forming new circuits important for recovery (Ward, 2017). This neuroplasticity takes place in surrounding perilesional as well as contralesional brain tissue (Cassidy & Cramer, 2017). Within this critical period, a level of spontaneous recovery of function occurs without any intervention. Spontaneous biological recovery is most feasible to study in preclinical models where rehabilitation can be withheld unlike human studies (Jeffers, Karthikeyan, and Corbett 2018). However, the potential of spontaneous biological recovery in patients has been conceptualized in a model of *proportional recovery* where motor recovery typically improves by ~70% from stroke onset (Prabhakaran et al., 2008). With the help of spontaneous recovery, the majority of motor recovery extends up to 3 months in humans (Cramer, 2008) and a month in rodents (Murphy & Corbett, 2009) before plateauing. Following stroke that results in upper limb deficits, the optimal timing and number of repetitions of rehabilitation for optimal motor recovery is still unclear in humans but insight can come from preclinical stroke research. In rodent models, rehabilitation is suggested to begin between day 5 and 14 post-stroke (**Figure 1**) (Murphy & Corbett, 2009). If started too early after stroke (<5 days) there is a risk of adverse effects for recovery such as blocking plasticity or exacerbating cell death (Krakauer, Carmichael, Corbett, & Wittenberg, 2012). Rehabilitative efficacy declines

over time and delayed rehabilitation in rats (>30 days) shows no benefit on behavioural recovery even at the structural level (Biernaskie, Chernenko, and Corbett 2004). In regards to repetitions, more is likely better. To see physiological changes in the motor cortex in rodents, several repetitions of 400 per daily session is required, far below the average of 32 per day in patients (Krakauer et al., 2012). Overall, to restore function and improve recovery of stroke survivors, current research goals are to enhance recovery within the critical period beyond what occurs spontaneously, to prolong or reopen the window of plasticity (Corbett et al., 2015). However, recent evidence suggests that intensive upper limb rehabilitation, even in the chronic post-stroke period, may produce functional gains (Ward, Brander, & Kelly, 2019).

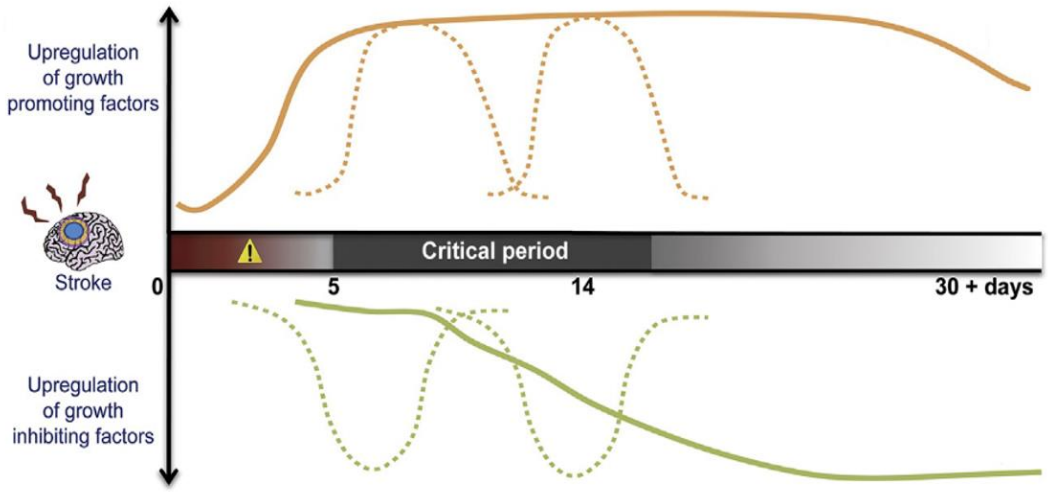

Figure 1. Critical periods of heightened plasticity post-stroke in rats. Time course based from focal ET-1 induced stroke in rodents. Initiation of rehabilitation and other interventions before day 5 may exacerbate damage and/or interfere with neuroplasticity processes. The critical period to begin therapy is between day 5 and 14 when there is steep upregulation of growth-promoting factors (orange line) followed by a delayed elevation of growth-inhibitory factors (green line). During this period some growth-promoting and growth-inhibitory genes are transiently upregulated (dashed lines). Early therapy within the critical period is best as efficacy diminishes over time. By day 30, inhibitory factors dominate which is not conducive to neuroplasticity or behavioural improvement. This critical period is likely different in humans where spontaneous recovery can last up to 3 months compared to 1 month in the rat. Figure modified from Murphy and Corbett 2009 and Corbett et al. 2015.

1.3 Exercise

Aerobic exercise before or early after stroke (24-48 h) is neuroprotective, reducing apoptosis, inflammation and stroke lesion volume (Austin, Ploughman, Glynn, & Corbett, 2014). Further, exercise is capable of stimulating repair processes and enhancing neuroplasticity to improve post-stroke recovery (Alcantara et al., 2018; Austin et al., 2014). The underlying neuroprotective and regenerative benefits of exercise are likely diverse, due to the widespread effects of exercise on different cell types, tissues and organs (Hawley, Hargreaves, Joyner, & Zierath, 2014). Some examples of the processes triggered by exercise important for stroke recovery include increased CBF, increased vascular density, upregulation of growth factors (eg. insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) (Austin et al., 2014; Nishijima, Torres-Aleman, & Soya, 2016; So et al., 2017). In particular, BDNF contributes to post-stroke recovery induced by both exercise (Ploughman et al., 2009) and drug therapy (Clarkson et al., 2011). It is also enhances motor learning and motor cortex plasticity important for motor stroke recovery (Mang, Campbell, Ross, & Boyd, 2013). In addition, aerobic exercise helps prime the brain to be more receptive to rehabilitation or task specific therapy (Mang et al., 2013). This combined therapy has shown to be very effective across other stroke therapies (eg. rehabilitation paired with growth factors (Jeffers et al., 2014) or environmental enrichment (Biernaskie & Corbett 2001)), a concept becoming emphasized in the field (Corbett et al., 2015). Nonetheless patients require a degree of aerobic conditioning, mobility and willingness to participate and reap these benefits of exercise (Billinger et al., 2014). To overcome these barriers to exercise rehabilitation, an intervention with a degree of overlaying mechanisms and benefits as exercise would be valuable.

1.4 Remote Ischemic Conditioning

Ischemic conditioning (IC) is a phenomenon in which brief periods of sublethal ischemia can offer protection against ischemia. For instance, natural IC may exist in patients who suffer transient ischemic attacks (TIA) prior to a stroke, as they are found to have less severe strokes, smaller infarcts (Wegener et al., 2004), and better functional outcomes (Moncayo, De Freitas, Bogousslavsky, Altieri, & Van Melle, 2000; Sitzer et al., 2004; Weber, Diener, & Weimar, 2011; Weih et al., 1999). Experimentally, IC was first described locally in the dog heart in 1986. Transient 5-minute occlusions of an artery supplying the heart prior to a sustained 40-minute occlusion was cardioprotective, resulting in less cardiac damage (Murry, Jennings, & Reimer, 1986). Despite the recognized benefits, performing IC on a vital organ is invasive and high-risk, reducing its potential for clinical application. The discovery of remote ischemic conditioning (RIC) facilitated the translation of IC into many clinical settings, such as cardiac surgery (eg. valve replacement, coronary artery bypass grafting, congenital heart disease), aortic and carotid artery surgeries and organ transplants (Candilio 2013; Le Page & Prunier 2015).

In RIC, blood flow is reduced in a non-vital organ or distal tissue such as skeletal muscle in a limb, by using a tourniquet or blood pressure cuff to occlude the vasculature supplying the tissue. Bouts of ischemia in the muscle are capable of inducing ischemic tolerance in distal vital organs (eg. heart or brain) to protect against a subsequent ischemic insult (Hess et al., 2015). This concept is similar to the effect of aerobic exercise, an established cardio- and neuro-protectant, and why RIC is sometimes considered an “exercise equivalent” or "exercise mimetic" (Ding et al., 2004; Williams et al., 2014; Zhang, Wu & Jia 2011; Hess et al., 2015).

The optimal RIC protocol (duration, timing and intensity) has yet to be determined. The two most widely used regimens are four repetitions of 5 minute occlusions/reperfusions (first used by

Murry et al. in 1986) and three repetitions of 10 minute occlusions/reperfusions. However, prolonged occlusion of 10 minutes is used less in the clinical setting and was found to abrogate protection compared to 2-5 minutes in a mouse model of myocardial infarction (Chen, Thakkar, Robinson, & Doré, 2018; Johnsen et al., 2016; Koch, Della-Morte, Dave, Sacco, & Perez-Pinzon, 2014). In the same study, RIC applied to one or two limbs was found to be equally protective (Johnsen et al., 2016). In humans, using an in vitro model of hypoxia-reoxygenation, it was found that arm versus thigh RIC had the same degree of cytoprotection (Dezfulian et al., 2017).

The signal originating in the limb from RIC that confers protection in the brain is not well understood, but three pathways (humoral, neural, and inflammatory) have been proposed, which are consistent across the temporal variants of RIC (Ezepue & Hess, 2017; Zhou et al., 2018). The humoral pathway is thought to be a primary mechanism of RIC. Transferring "RIC" blood dialysates, even between species, offers cardioprotection to "nonRIC" organisms (Shimizu et al., 2009). Dialysates from individuals who underwent vigorous exercise or RIC offered equal protection in the rabbit heart (Michelsen et al., 2012). RIC is ineffective without reperfusion intervals between occlusions, highlighting the importance of blood flow in mitigating protection. Shear stress on blood vessels during reperfusion releases nitric oxide (NO) into circulation, triggering vasodilation that likely contributes to increased cerebral blood flow (Rassaf et al., 2014). Postulated blood-borne mediators of RIC include nitrite (derived from NO), stromal cell derived factor-1 α, microRNA-144, and IL-10 (Cai, Parajuli, Zheng, & Becker, 2012; Davidson et al., 2013; Jing Li et al., 2014; Rassaf et al., 2014). Interestingly, the humoral system depends on innervation by peripheral nerves. If nerves are transected or pharmacologically blocked, ischemic protection as well as humoral factors are attenuated (Hess, Hoda, & Bhatia, 2013).

Patients with peripheral neuropathy also do not reap the benefits from RIC, supporting the importance of the afferent neural pathway in RIC (Jensen, Støttrup, Kristiansen, & Bøtker, 2012). During RIC, sensory nerves are believed to be stimulated by the local release of autacoids (eg. adenosine and bradykinin) released from ischemia-reperfusion in muscle (Schoemaker & van Heijningen, 2000; Steensrud et al., 2010). Lastly, a systemic anti-inflammatory state induced by RIC is modulated by the suppression of proinflammatory genes and upregulation of anti-inflammatory genes in immune cells (Zhou et al., 2018). With repeated RIC, macrophages and neutrophils are reduced over time and leads to a long term state of reduced inflammation (Wei et al., 2011).

After each bout of RIC, there are two proposed waves of protection (**Figure 2**) (Zhao et al. 2018; Sommer 2008). The first wave is immediate and short (beginning at 0 h until 2 h). During this time, there are changes in ion permeability, proteins are phosphorylated and protective mediators are released (Ren et al., 2008). Examples include NO, microRNA-144, Erk/Akt, hypoxia inducible factor (HIF), etc. The second wave is delayed and longer lasting (beginning at 12-24 h until 48-72 h) (Kuzuya et al., 1993). Changes in inflammation, endothelial function and protein expression (eg. heat shock proteins (HSP), nitric oxide synthase (NOS), Erk/Akt)) are expected during this time (Kis, Yellon, & Baxter, 2003; Yellon & Baxter, 1995).

Clinically, RIC as an appealing therapy because of its easy implementation and low cost. RIC has been explored in the clinical setting since 2000 and is growing as a strategy for ischemic prevention as an adjunctive therapy (Gunaydin et al., 2000; Le Page & Prunier, 2015). RIC is safe and well tolerated in patients who received bilateral arm RIC twice a day for 300 days and is well-tolerated by 97% of participants (Bai & Lyden, 2015; Gonzalez, Connolly, Dusick, Bhakta, & Vespa, 2014; Koch, Katsnelson, Dong, & Perez-Pinzon, 2011; Meng et al., 2012).

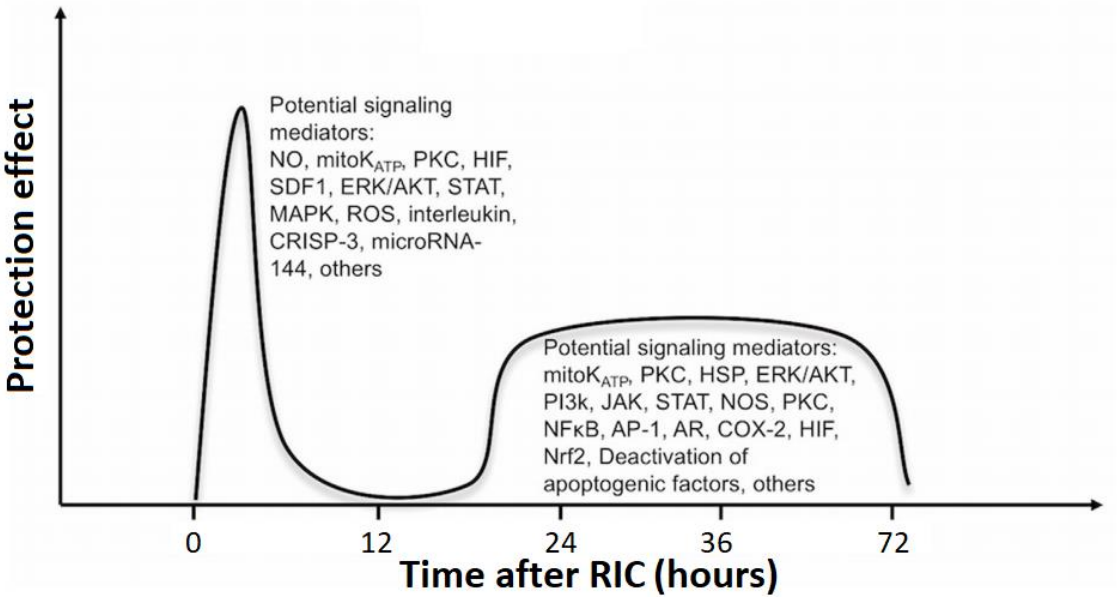

Figure 2. Proposed mediators involved in the two waves of protection following remote ischemic conditioning (RIC). The first wave is immediate and short (beginning at 0 h until 2 h) and the second wave is delayed and longer lasting (beginning at 12-24 h until 48-72 h). Abbreviations: AR, aldose reductase; AP-1, activator protein 1; COX-2, cyclooxygenase-2; CRISP-3, cysteine-rich secretory protein 3; NOS, nitric oxide synthase; ERK/AKT, extracellular signal regulated kinase/protein kinase B; HIF, hypoxia-inducible factor; HSP, heat shock protein; JAK, Janus kinase; KATP, ATP-sensitive potassium channel; MAPK, mitogen-activated protein kinase; Mito, mitochondria; NFκB, nuclear factor κB; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor; PI3k, phosphoinositide-3 kinase; PKC, protein kinase C; ROS, reactive oxygen species; SDF1, stromal cell-derived factor 1; STAT, signal transducer and activator of transcription. Figure modified from Zhou et al., 2018.

1.5 Neuroprotective Effect of Remote Ischemic Conditioning

The majority of preclinical RIC applications involving stroke have studied its neuroprotective properties to reduce the size of the injury. RIC can take place at three time points relative to stroke to offer neuroprotection: (1) preconditioning (RIpreC) occurring immediately to hours (sometimes days) before stroke, (2) perconditioning (RIperC) occurring during the ischemic stroke but before reperfusion and (3) postconditioning (RIpostC) occurring immediately after reperfusion and onwards (**Figure 3**). All RIpreC and RIpreC neuroprotection studies in rats are summarized in **Table 1 and 2**. With as little as one session of RIC, neuroprotection is evident.

Immediate RIpreC (Wei et al. 2012; Ren et al. 2008) and RIpostC (Ren et al., 2009), reduces infarct volume by 80% and 67%, respectively (**Table 1 and 2**). Despite apparent neuroprotective effects, delivering RIC immediately before, during, or after a stroke is logistically a challenge for clinical interpretation, yet many preclinical studies continue to use this narrow treatment window (**Table 1 and 2**). When RIC is delivered at a longer interval after stroke, it appears that neuroprotective effects exist, albeit to a lesser degree. For instance, in rats one session of delayed 12 hour RIpreC or 3 hour RIpostC resulted in infarct reductions of ~52% (Ren et al., 2008) and 43% (Ren et al., 2009), respectively.

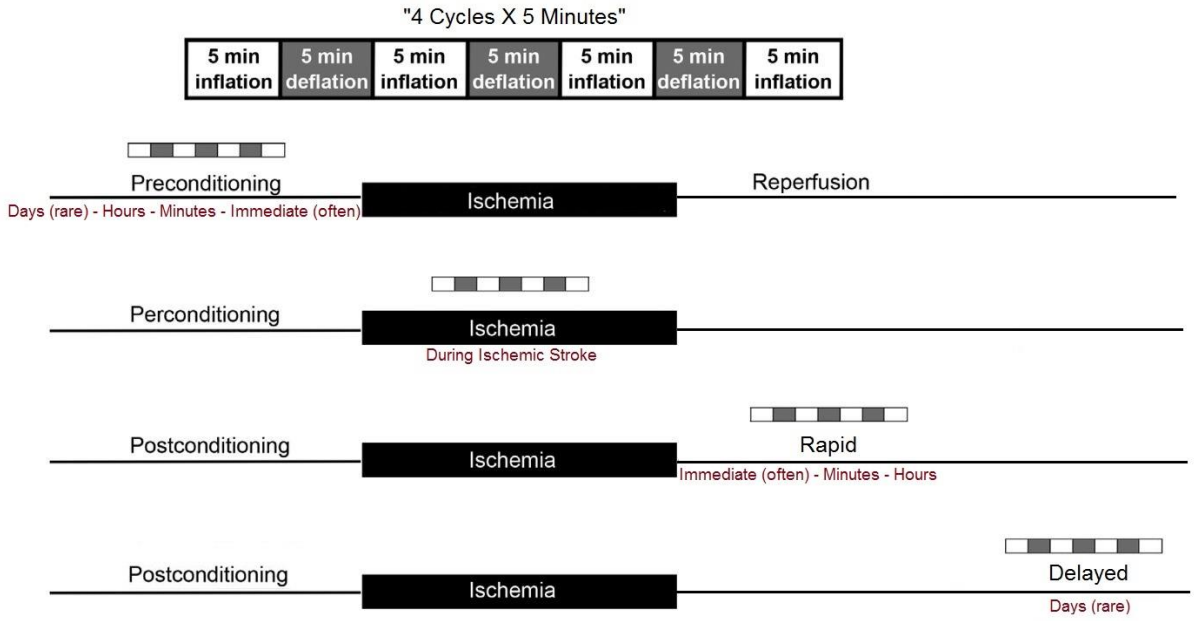

Figure 3. Temporal variants of remote ischemic conditioning (RIC) relative to ischemic stroke. The most common RIC protocol is 4 cycles of alternating 5-minute cuff inflations (causing ischemia) and 5-minutes of cuff deflation (allowing reperfusion). Preconditioning (RIpreC) occurs at any time before stroke but most often within an hour before. Perconditioning (RIperC) occurs during the ischemic stroke before reperfusion. Postconditioning (RIpostC) can be rapid or delayed. Rapid RIpostC takes place during reperfusion and often within the first hour after stroke and promotes neuroprotection. In contrast, delayed RIpostC delivered days after stroke (once cell death is complete) may benefit regenerative processes beneficial for stroke recovery. Figure modified from Hess, Hoda, and Bhatia 2013.

In addition to RIC timing relative to stroke, it is also important to consider the time and method in which lesion volumes were measured relative to stroke or last RIC bout. Most RIC studies assess infarct volume1-2 days post-stroke and are quantified using triphenyltetrazolium chloride (TTC) staining (**Table 1 and 2**). TTC stains non-infarcted tissue with functioning mitochondria red but is susceptible to overestimating lesion volumes (Benedek et al., 2006; Sommer 2019). Furthermore, RIC has only been applied in the MCAO model of stroke, which is a weakness for clinical translation due to the heterogeneous nature of stroke (Corbett et al., 2017; Edwardson et al., 2017). Importantly however, it was found that RIC treatment did not mitigate the effects of tPA when given together (using an embolic MCAO model) (Hoda et al. 2012; Hougaard et al. 2014). Another shortfall within the literature is that females have not been well-represented. Only a single group at Chengdu Medical College in China have studied RIpostC in female rats and none for RIpreC (**Table 2**) (Li et al., 2018; Li et al., 2015). Importantly, no study has carried out a direct comparison of RIC neuroprotection in males versus females.

Although stroke incidence and prevalence of stroke is lower in women (Peltonen et al., 2015) and females have smaller stroke lesions than males (Alkayed et al., 1998), the mechanistic reasonings for these sex differences are still unclear (Peltonen et al., 2015). This strongly supports STAIR guidelines insisting that sex-differences of any neuroprotective intervention be investigated prior to clinical implementation (Stroke Therapy Academic Industry Roundtable (STAIR), 1999). In addition, evidence suggests neuroprotective mechanisms of RIC (eg. enhanced Akt (Zhang, Wang, & Bi 2017; Hoda et al., 2012) and VEGF signaling (Ueno et al., 2016), increased CBF and angiogenesis (Khan et al., 2018) and induction heat shock proteins (Xia, Ding, Zhang, & Feng, 2017)) overlap with those induced by aerobic exercise (Hess et al.,

2015; Zhao et al., 2018; Michelsen et al., 2012). Interestingly, exercise may also have sex-specific effects in vascular function and structure (Green et al., 2016). For instance, chronic increases in blood flow (eg. exercise, repetitive RIC) mediated by shear stress and NO for structural remodelling (eg. increased vessel diameter, angiogenesis) may be estrogen-dependent as endothelial cells responsible for these changes have an abundance of estrogen receptors (E2 and ERα) (Tarhouni et al., 2013). Consequently, CBF also tends to be higher in females (Peltonen et al., 2015; Rodriguez, Warkentin, Risberg, & Rosadini, 1988), perhaps because vasodilation maintenance of endothelial cells from estrogen. Sex-differences in cell death pathways also exist. Interestingly, females may be insensitive to NO mediated cell death pathway compared to males (McCullough, Zeng, Blizzard, Debchoudhury, & Hurn, 2005) and the predominant cell-death pathway in females depend on caspase release from dysfunctional mitochondria (Liu et al., 2009). Since CBF, NO and improved mitochondria function (Slagsvold, Rognmo, Høydal, Wisløff, & Wahba, 2014) may be mediators increased by RIC, females may be further protected with less cell-death and superior maintenance of endothelial cells to readily adapt to ischemia resulting in extended neuroprotection with reduced infarcts than males.

Table 1. Neuroprotection of remote ischemic pre-conditioning (RIpreC) in rats. Abbreviations: MCAO= middle cerebral artery occlusion, BCAO=bilateral common carotid artery occlusion, SD=Sprague-Dawley rat, S.E.M =standard error of mean, TTC =triphenyltetrazolium chloride.

Start RIpreC	RIC Frequency: # Days (cycles X duration of inflation)	Occlusion Method	# limbs	% Change Infarct Size	Effect Size (d)	Time Post-stroke Measure Infarct + Method	Mean Infarct Volume (mm3 or %) +/- Error (SEM), (n)		Type MCAO + Occlusion Time (minutes)	Rat Species + Sex	Reference
							Control	RIC			
0hr	1 (3 X15min)	Femoral artery	1	↓ ~80%	5.65	@2d with TTC	~48 ±3 % (6-7)	~10 ±2 % (6-7)	Permanent Distal + 30 BCAO	SD Male	Wei et al., 2012
				↓40%	3.78	@2m with Cresyl Violet	~42 ±4 % (6-7)	~22 ±4 % (6-7)			
2d	1 (3 X 15min)	Femoral artery	1	↓ ~44%	0.93	@2d with TTC	~41 ±2 % (6)	23 ±11 % (6)	Permanent Distal + 30 BCAO	SD Male	Ren et al., 2008
12hr				↓ ~52%	1.65		~52 ±8 % (6)	25 ±5 % (6)			
0hr				↓ 80%	1.99		48 ±8 % (6)	10 ±9 % (6)			
0hr	1 (3 X 15min)	Femoral artery	1	↓73%	4.37	@2d with TTC	25 ±3 % (6)	~7 ±5% (6)	Permanent Distal + 30 BCAO	SD Male	Xu et al., 2017
1hr	1 (3 X 5min)	Tourniquet	1	↓ 60%	2.40	@1d with TTC	235 ±20 to mm^3 (10)	94 ±22 mm^3 (6)	Proximal 90	Wistar Male	Bonova et al., 2015
1hr	1 (4 X 5min)	Tourniquet	2	↓~49%	2.51	@3d with TTC	~55 ±2 % (8)	~28 ±5% (8)	Proximal 90	SD Male	Chen et al., 2018
				↓~44%	3.79		~45 ±2 % (6)	~25 ±5 % (6)		SD Male	
0hr	1 (3 X 15min)	Femoral artery	1	↓~48%	1.77	@2d with TTC	~29 ±2 % (6)	~15 ±4% (6)	Permanant +30 BCAO	SD Male	Jin et al, 2016
0hr	1 (3 X 15min)	Femoral artery	1	↓~46%	4.12	@2d with TTC	~26 ±0 % (6)	~14 ±2% (6)	Permanent Distal + 30 BCAO	SD Male	Liu et al., 2018
3d	3 (3 X 10min)	Femoral artery	2	↓ 40%	2.55	@1d with TTC	43 ±3 % (8)	25 ±2 % (8)	Proximal120	SD Male	Zhang et al., 2012
1hr	1 (3 X 5min)	Touniquet	1	↓~34%	1.84	@1d with TTC	~380 ±5 mm^3 (16)	~250 ±4 mm^3 (16)	Proximal120	SD Male	Hu et al., 2012
0hr	1 (4 X 5min)	Tourniquet	1	↓ ~24%	0.62	@1d with TTC	~240 ±40 mm^3 (12-14)	~170 ±50 mm^3 (12-14)	Proximal 120	SD Male	Hahn et al., 2011
1d?	14 (3 X 10min)	Touniquet	2?	↓~17%	0.71	@1d with TTC	~52 ±5 % (8)	~43 ±4 % (8)	Proxiamal 90	SD Male	Ren et al., 2017
1hr	1 (4 X 5min)	Touniquet	2	↓~12%	0.44	@3d with TTC	~250 ±25 mm^3 (12)	~220 ±12 mm^3 (12)	Proximal 90	SD Male	Liu et al., 2016
		Weighted Average Effect Size			**3.61 ± 2.2 SD**						

Table 2. Neuroprotection of remote ischemic post-conditioning (RIpostC) in rats. Abbreviations: MCAO= middle cerebral artery occlusion, BCAO=bilateral common carotid artery occlusion, SD=Sprague-Dawley rat, S.E.M =standard error of mean, TTC =triphenyltetrazolium chloride.

Start RIpostC	RIC Frequency: # Days (cycles X duration of inflation)	Occlusion Method	# limbs	% Change Infarct Size	Effect Size (d)	Time Post-stroke Measure Infarct + Method	Mean Infarct Volume (mm3 or %) +/- Error (SEM), (n)		Type MCAO + Occlusion Time (minutes)	Rat Species + Sex	Reference
							Control	RIC			
0hr	1 (3 X 15min)	Femoral artery	1	↓67%	4.45	@2d with TTC	44 ±1 %(6)	15 ±3 % (11)	Permanent Distal + 30 BCAO	SD Male	Ren et al., 2009
3hr				↓43%	1.48		44 ±1 % (6)	25 ±6 % (8)			
6hr				↓~28% n.s.	1.05		44 ±1 % (6)	~32 ±7 % (6)			
0hr				↓24%	1.41	@2d with Cresyl Violet	58 ±7 % (8)	45 ±4 % (10)			
0hr				↓0% n.s.	0.00	@2mo with Cresyl Violet	~44 ±3 % (11)	~44 ±4 % (8)			
0hr	1 (3 X 10min)	Femoral artery	2	↓66%	2.85	@1d with TTC	35 ±4 % (8)	12 ±1 % (5)	Proximal 90	SD Male	Ren et al., 2011
0hr	1 (3 X 10min)	Femoral artery	2	↓59%	4.50	@3d with TTC	77 ±5 % (5)	32 ±5 % (5)	Proximal 90	SD Male	Huang et al., 2017
0hr	1 (3 X 10min)	Tourniquet with bands in tubing	2	↓50%	7.37	@1d with TTC	30 ±1 % (16)	15 ±0 % (16)	Proximal 60	SD Female	Li et al., 2015
				↓~33%	-	@3d with TTC	~33 ±? % (16)	22 ±0 % (16)			
0hr	1 (3 X 5min)	Cuff	1	↓47%	2.28	@1d with TTC	28 ±2 % (5)	15 ±3 % (5)	Proximal 90	SD Male	Cheng et al., 2014
0hr	1 (3 X 15min)	Femoral artery	1	↓47%	3.31	@1d with TTC	32 ±2 % (6-8)	17 ±2 % (6-8)	Proximal 120	SD Male	Chen et al., 2014
3hr	1 (3 X 15sec)	Femoral artery	2	↓5% n.s.	1.13	@3d with TTC	64 ±1 % (8)	60 ±1 % (8)	Proximal 90	SD Male	Sun et al., 2012
	1 (3 X 5min)			↓~33%	5.94		64 ±1 % (8)	~43 ±1 % (8)			
	1 (3 X 8min)			↓~34%	5.50		64 ±1 % (8)	~42 ±1 % (8)			
6hr	1 (3 X 15sec)			↓~32%	4.42		64 ±1 % (8)	~44 ±2 % (8)			
	1 (3 X 5min)			↓46%	7.25		64 ±1 % (8)	35 ±1 %(8)			
	1 (3 X 8min)			↓~39%	7.07		64 ±1 % (8)	~39 ±1 % (8)			
0hr	1 (4 X 5min)	Tourniquet	1	↓~46%	1.69	@1d with TTC	~41 ±1 % (6)	~22 ±2 % (6)	Proximal 120	SD Male	Wang et al, 2016
2d	21 (3 X 10min)	Tourniquet	1?	↓45%	5.88	@21d with MRI	146 ±4 mm^3 (10)	81 ±3 mm^3 (10)	Proximal 90	SD Male	Liang et al., 2018
0hr	1 (3 X 10min)	Tourniquet	2	↓42%	0.82	@1d with TTC	~31 ±5 % (8)	~17 ±4 % (8)	Proximal 60	SD Female	Li et al., 2018
1d	7 (3 X 10min)	Tourniquet	2	↓~44%	2.76	@1w with Cresyl violet	~41 ±3 % (5)	~23 ±3 % (5)	Proximal 90	SD Male	Ren et al., 2015
	14 (3 X 10min)			↓~43%	3.26	@2w with Cresyl violet	~42 ±2 % (5)	~24 ±3 % (5)			
0hr	1 (3 X 5min)	Femoral artery	1	↓~44%	1.41	@1d with TTC	~27 ±2 % (6)	~15 ±2 % (6)	Proximal 90	SD Male	Chen et al., 2016
1hr				<44%	-		-	-			
3hr				<44%	-		-	-			
0hr	1 (3 X 10min)	Femoral artery	2	~32%	1.06	@1d with TTC	~28 ±3 % (6)	~19 ±2 % (6)	Proximal 120	SD Male	Zhang et al., 2017
0hr	1 (3 X 10min)	Femoral artery	2	↓31%	1.69	@2w with Cresyl violet	51 ±5 % (6)	35 ±2 % (6)	Proximal 90	SD Male	Wang et al., 2018
0hr	1 (3 X 10min)	Femoral artery	2	↓ ~26%	2.87	@1d with TTC	~50 ±2% (6)	~37 ±2 % (6)	Proximal 120	SD Male	Liu et al., 2014
		Weighted Average Effect Size			**2.22 ± 1.5 SD**						

1.6 Remote Ischemic Conditioning and Neuroplasticity

All temporal variants of RIC (Pre, Per, and Post) have been demonstrated to be neuroprotective following MCAO in animal models of stroke (Hoda et al., 2012; Ren et al., 2008; Ren et al., 2009), however, the potential of delayed RIpostC beginning hours to days after stroke (**Figure 3**) and its potential for promoting neuroplasticity and stroke recovery is relatively unknown (Liang et al., 2018; Sutter et al., 2018). Despite the existence of a critical period following stroke when the initiation of rehabilitation seems to most effective, stroke survivors are extremely sedentary spending over 50% of their time in bed with minimal physical activity (Bernhardt, Dewey, Thrift, & Donnan, 2004). During this time, stroke survivors become physically deconditioned, experience muscle atrophy of the affected limb (Ivey, Macko, Ryan, & Hafer-Macko, 2015) and develop a reduced capacity for exercise (MacKay-Lyons & Makrides, 2002). As a result, potential functional gains from physical rehabilitation are compromised (Hyngstrom et al., 2018). Despite knowing the benefits of exercise for stroke recovery and even the synergistic effects it can have with other forms of rehabilitation (Mang et al., 2013), the high prevalence of post-stroke fatigue and depression is a significant barrier preventing patients from engaging in exercise to help prevent physical deterioration (MacIntosh et al., 2017). More feasible interventions that can take advantage of this down time post-stoke to help prevent physical decline and promote stroke recovery are necessary. For example, RIC of the thigh can delay muscle fatigue in the arm (Barbosa et al., 2015) and a single session of RIC of the thigh (5 cycles of 5 minute inflations/deflations) can increase muscle strength through improving neural activation in the knee (Hyngstrom et al., 2018). In addition, RIC shares important neural repair and regenerative signals important for stroke recovery (Murphy & Corbett, 2009) as exercise (Alcantara et al., 2018; Austin et al., 2014), such as VEGF (Ueno et al., 2016) and BDNF

(Ramagiri & Taliyan, 2017). This evidence highlights the potential of chronic RIpostC (C-RIpostC) occurring repetitively for periods of weeks or months after stroke, possibly mimicking exercise, as a promising therapy for sedentary stroke survivors.

Some evidence demonstrates that C-RIpostC can promote angiogenesis and vascular remodeling to increase blood flow to damaged brain tissue, similar to the effect of exercise (Khan et al., 2018; Ma, Qiang, & He, 2013). Angiogenesis stimulates important endogenous recovery mechanisms such as synaptogenesis, synaptic plasticity, and neurogenesis (Ergul, Alhusban, & Fagan, 2012). C-RIpostC beginning 3 or 7 days after cerebral hypoperfusion lasting 3-4 weeks improves cognitive recovery along with increased CBF, angiogenesis marked by greater capillary densities and angiogenic markers (eg. CD31) in the brain, and arteriogenesis marked by collateral vessels (Khan et al., 2018; Changhong Ren et al., 2018). In addition, elevated expression of phosphorylated endothelial nitric oxide synthase (p-eNOS) was found in the hippocampus (Ren et al. 2018). eNOS is considered to be a key factor maintaining the integrity of vascular endothelial cells (eg. proliferation and maintenance) and mediates angiogenesis following ischemia (Abu-Amara et al., 2011). Phosphorylation of eNOS to p-eNOS triggers the production of NO, which has a diversity of roles including the stimulation of VEGF, angiopoietins and fibroblast growth factor. Furthermore, if eNOS is inhibited with a NOS inhibitor (eg. L-NAME), proangiogenic, as well as cognitive, gains are attenuated (Changhong Ren et al., 2018). Interestingly, daily C-RIpostC for 3 weeks compared to 4 months resulted in equivalent recovery measured by CBF, memory, and balance at 6 months (Khan et al., 2018). While C-RIpostC has been applied in the context of cognitive recovery using global cerebral hypoperfusion, it is uncertain if and how these proangiogeneic changes may influence motor stroke recovery or a more focal injury. One study performing C-RIpostC of 3 cycles of 10-

minutes for 3 weeks in a rat middle cerebral artery occlusion (MCAO) model improved motor function on the ladder and beam tests. However, as delayed C-RIpostC only started 2 days after injury and reduced infarct size, it is unclear if neuroprotection reducing the infarct accounts for the observed motor recovery (Liang et al., 2018).

Important growth and plasticity factors for stroke recovery, such as BDNF and growth associated protein 43 (GAP43) (McDonald, Hayward, Rosbergen, Jeffers, & Corbett, 2018; Ploughman et al., 2009), have also been observed to increase following RIC (Mizutani, Sonoda, Yamada, Beppu, & Shimpo, 2011; Ramagiri & Taliyan, 2017). Rats with MCAO given immediate RIpostC for a single session have elevated levels BDNF in brain tissue, however, it is unknown if BDNF would also be elevated chronically following delayed C-RIpostC (Ramagiri & Taliyan, 2017). GAP43 has also been shown to be increased in the hippocampus at later stages (4 weeks of delayed C-RIpostC beginning 3 days following cerebral hypoperfusion) in rats (Mizutani et al., 2011; Changhong Ren et al., 2018). Another important protein stimulated by RIC relevant for stroke recovery is protein kinase B, also known as Akt. The importance of Akt, and its phosphorylated form, have been emphasized with other stroke interventions such as environmental enrichment, exercise, and atorvastatin (Clarkson et al., 2015; Elewa et al., 2009). Additionally, through the activation of cAMP response element binding (CREB) protein, pAKT promotes neuronal survival by upregulating BDNF (Zhang et al., 2013). Although not yet studied in stroke recovery or using C-RIpostC, all three temporal variants of RIC show prolonged p-AKT at the endothelium that increases the eNOS/NO pathway to maintain vascular homeostasis (Zhou et al., 2018).

Overall RIC as an intervention has several promising neurovascular and neurogenic mechanisms, similar to those established with exercise that have been linked to brain repair and recovery after

stroke. However, it is unknown if delayed C-RIpostC after focal ischemia will stimulate these similar factors and how it may influence motor stroke recovery.

1.7 Rationale

RIC is a non-invasive procedure that may mimic intrinsic neuro-protective and -regenerative pathways, similar to aerobic exercise that may benefit stroke survivors (rather than a single target approach). RIC may be most advantageous for stroke survivors who are unable to reap the benefits of exercise due to aerobic deconditioning, extreme fatigue or depression. However, RIC efficacy has only been demonstrated in a single preclinical stroke model (MCAO) which is a shortcoming for clinical translation. Since sex differences exist in stroke and exercise, it is of interest to determine if the efficacy of RIC is the same in both sexes. Further, to date the delivery of RIC has occurred at times that may not be feasible for humans (pre-stroke or acutely post-stroke).

1.7.1 Hypothesis

i) RIC will be neuroprotective in the ET-1 stroke model.

ii) RIC will be more effective in females than males due to inherent cerebrovascular benefits (eg. higher CBF than males).

iii) RIC will be most effective before stroke and show some efficacy in promoting stroke recovery.

1.7.2 Objectives

i) To determine if RIC is neuroprotective in a more clinically translational model of stroke (ET-1).

ii) To assess if sex differences exist in RIC neuroprotection.

iii) To explore the potential of RIC when delivered at clinically feasible times.

MATERIALS AND METHODS

2.1 Animals and Experimental Design

A total of 129 Sprague-Dawley rats (88 females and 41 males) were acquired from Charles River Laboratories (Montreal, Quebec). All rats were habituated for a week upon arrival. In *Experiment 1*, RIC was delivered either ~18 hours before stroke for *RIpreC* (**Figure 4a**) or 4 hours after stroke for *RIpostC* (**Figure 4b**). In *Experiment 2*, (*C-RIpostC*), RIC was delivered daily for 4 weeks beginning at day 5 post-stroke. Rats arrived at the animal facility weighing 250-275g in *Experiment 1* (1week pre-stroke) and 200-250g in *Experiment 2* (3 weeks pre-stroke) (**Table 3**). No rats died of surgery complications. Rats were pair-housed on an 8am-8pm reverse light cycle with *ad libitium* access to 18% protein rodent diet (Teklad Global Diets). All experimental procedures were approved by the University of Ottawa Animal Care Committee and in accordance with the Guidelines of the Canadian Council of Animal Care.

Table 3. Number of male and female Sprague-Dawley rats used per experiment.

Experiment	Females			Males		TOTAL
	RIC	Control	Sham	RIC	Control	
1: RICpreC	11	10	-	11	10	42
1: RIpostC	11	10	-	10	10	41
2: C-RIpostC	20	20	6	-	-	46

For all experiments, MRI occurred 24 hours after stroke and rats were sacrifice 48 hours after their last RIC session. In *Experiment 2*, Animals were tested on the Montoya staircase, cylinder and tapered beam at (1) pre-stroke (-3 days post-stroke), (2) between day 3-5 post-stroke (3) after 7 days of RIC (12 days post-stroke and (4) 28 days of RIC (33 days post-stroke) (**Figure 4c**). Prior to stroke, training for the Montoya staircase took 2 weeks (daily from week -2 to 0). One RIC rat died during the first day of C-RIpostC treatment.

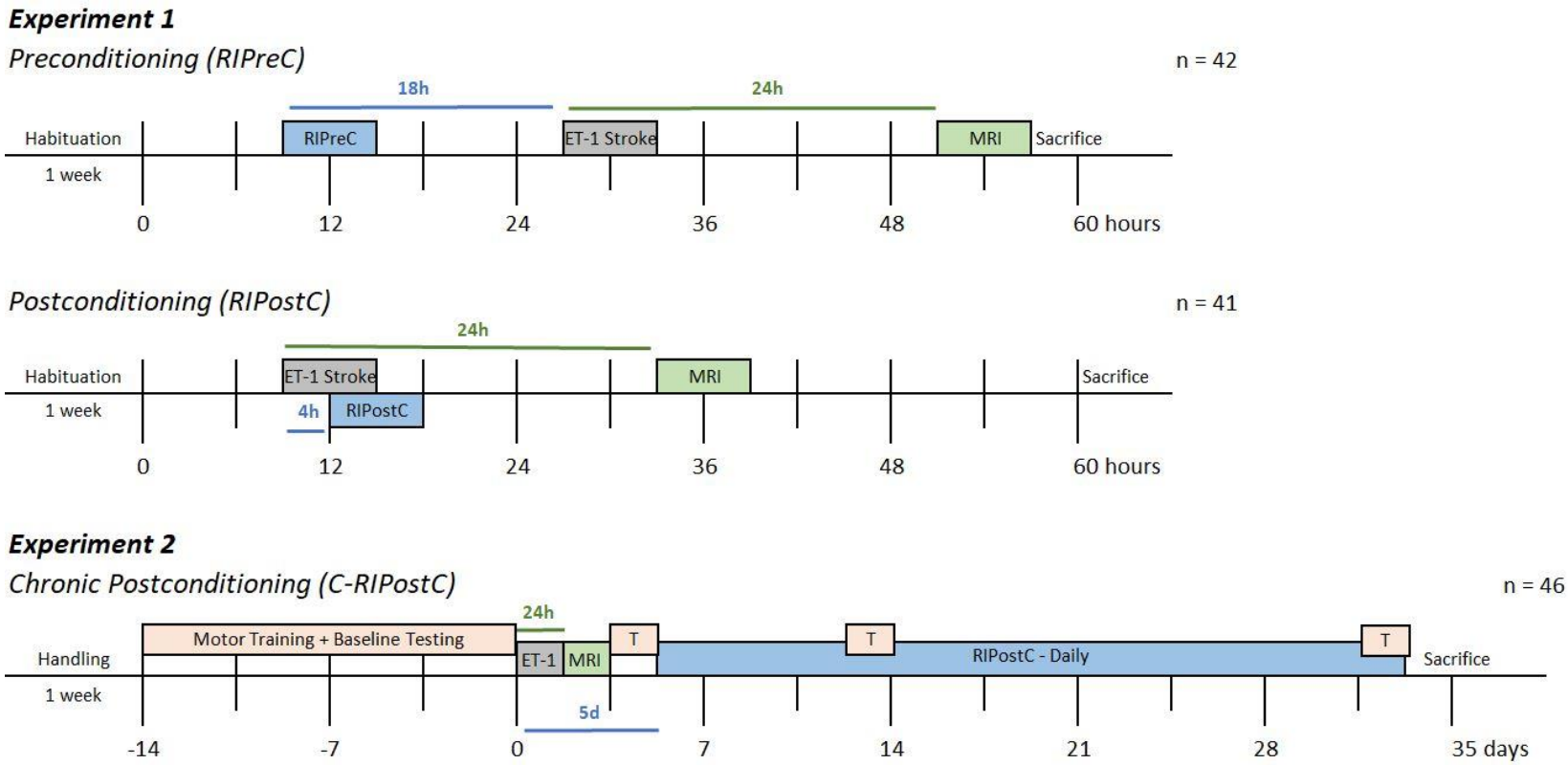

Figure 4. Experimental timelines for Experiment 1 and 2. In *Experiment 1*, preconditioning was delivered 18 hours before stroke and postconditioning was delivered 4 hours after stroke. Both delivery times for RIC in *Experiments 1* are at clinically feasible times relative to stroke and within the neuroprotection window. Neuroprotection was assessed by MRI infarct volume at 24 hours post-stroke (green). In *Experiment* 3, RIC began 5 days post-stroke, falling outside the neuroprotection window and in the biological recovery phase. However, MRI still occurred at 24 hours post-stroke to assess infarct volume prior to RIC intervention (green). Motor recovery testing (T) was measured at baseline (before stroke and RIC), 5 days post-stroke (before RIC), after 7 days of RIC (12 days post-stroke) and 28 days of RIC (33 days post-stroke (pink) Motor tests included: the Montoya staircase, tapered beam and cylinder. Additionally, gait was assessed after 4 weeks of RIC treatment. RIC parameters used for all experiments were 4 cycles of 5-minute inflations/deflations (total 40 minutes) while under isoflurane anesthesia. In the *Experiment 1* (Neuroprotection Phase), RIC was only performed once compared to the *Experiment 2* (Recovery Phase), where RIC was delivered chronically (repeated daily for 28 days (33 days post-stroke)) (blue).

2.2 Induction of Endothelin-1 Stroke

All 123 rats received focal ischemic strokes using the ET-1 stroke model (Karthikeyan et al., 2019; Windle & Corbett, 2005) while 6 rats received a sham procedure. Rats were fasted overnight (~16 hours) prior to surgery. Rats were anesthetized with 4% isoflurane with oxygen flow of 1.6L/min for induction. During induction, rats were prepared for surgery. Heads were shaved and the skin was wiped with a preoperative skin antiseptic (SoluPrep, 3M). Saline (0.9% NaCl, Baxter) was injected subcutaneously to prevent dehydration during surgery. To prevent dry eyes, tear-gel (Optixcare) was applied and a topical anesthetic (2% Xylocaine, Aspen) was applied in the ears to reduce any discomfort from ear bars during surgery.

While in a stereotaxic apparatus, a small incision was made in the scalp of the rat. Two holes approximately 1mm diameter were made in the skull using a motorized drill. A Hamilton syringe (Hamilton, 80366) was used to slowly (250 nl/min) inject endothelin-1 (400.0 pmol/µl, 1.0 µl/hole, Abcam, AB120471) in the forelimb sensorimotor cortex (Bregma coordinates: 0.0mm AP, ±2.5mm ML, -1.7mm DV and +2.3mm AP, ±2.5mm ML, -1.7mm DV). For *Experiment 1,* all strokes were directed to the left cortex. In *Experiment 2,* strokes were induced in the cortex opposite to the dominant limb (determined from pre-stroke Montoya staircase training). Body temperature was regulated at ~37°C during surgery with heating blankets and isoflurane was maintained at 1.5-2.0%. Following injection, the wound was sutured (Monosof suture size 4-0, Covidien, SN662), topical anesthetic cream (2% Bupivacaine, Chiron) was applied and the animal was returned to an incubator at 37°C to recover. Upon wakening rats received a subcutaneous injection of buprenorphine analgesic (0.05ml/kg, Chiron) and a second application of bupivacaine 4 hours later.

2.3 Delivery of Remote Ischemic Conditioning

The RIC protocol consisted of 5 minutes of inflation and 5 minutes of deflation over 4 cycles. Anesthetized rats were induced with 5% isoflurane and maintained at 3% with an oxygen flow of 2L/min. Using a 10-piece nozzle manifold, up to 10 rats (5 RIC and 5 non-RIC) were anesthetized at once. Tear-gel was applied to eyes. Hindlimbs contralateral to the lesioned hemisphere were shaved and neonatal blood pressure cuffs (size 1, 3-6cm, Dispomed Veterinary Instruments) were placed above the knee to receive RIC. Prior to beginning RIC, pulse sensors were placed on the animals footpads below the cuffs. To verify limb occlusion, during RIC (~40 minutes) both the foot pulse and oxygen saturation was monitored with a LifeSense Vet pulse oximeter (Nonin Medical) and automatically collected using custom acquisitions software on a Raspberry Pi microcomputer (see section 2.3.1 for the automation of pulse oximeter data collection). An automatic pump (see section 2.3.2 for details) was set with the following timing: time high pressure:300s, time low pressure:300s, trials:4, psi:3.3, see section 2.3.2). The blood pressure cuffs simultaneously underwent 4 cycles of 5 minutes of inflation and deflation at approximately 170mmHg (3.3psi), just above the blood pressure of anesthetized rats to occlude blood flow (Brandli, 2015) (**Figure 5**). Due to the daily use of anesthesia, rats were monitored frequently for signs of distress (activity level, hydration, hair coat, attitude and posture) and body mass were recorded twice per week to ensure total body mass loss did not exceed 15%.

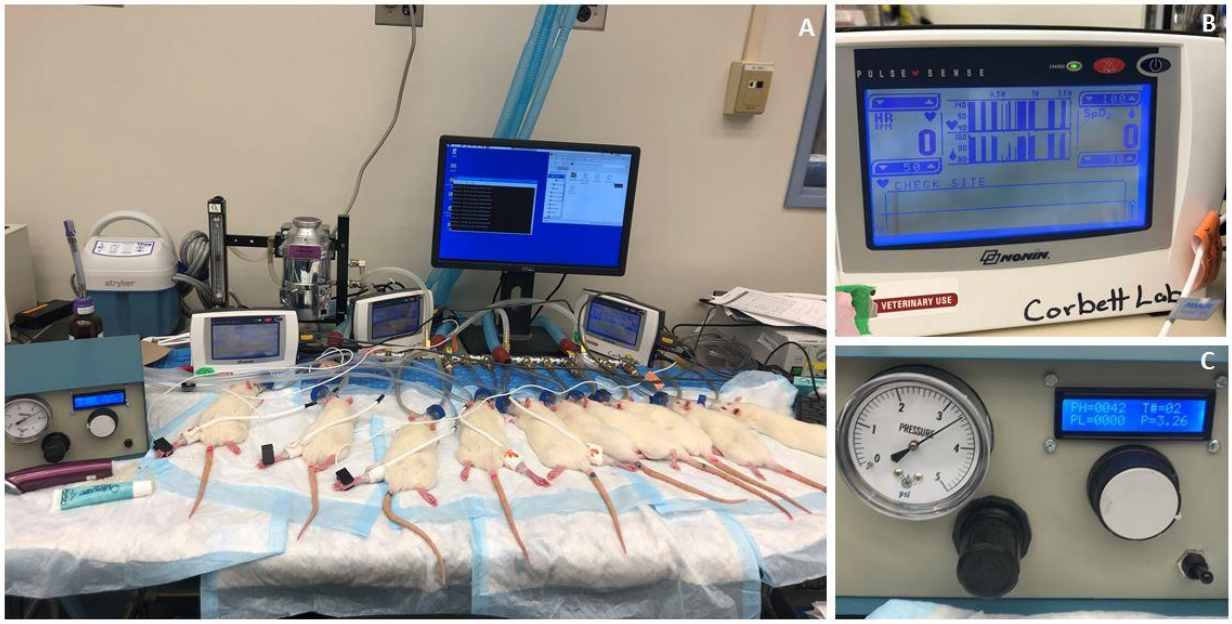

Figure 5. Simultaneous remote ischemic conditioning (RIC) of rat hindlimbs using programmable cuffs..(A) Representative image of rats anesthetized with isoflurane. The RIC protocol was set to automatically perform 4 cycles of 5-minute inflations/deflations (40 minutes total time) using the programmable cuff pump (left hand side and C). Pulse-oximeter sensors were placed on the foot pads below the cuffs of 3 RIC rats for the entire protocol. Using a raspberry pi, foot pulse and oxygen saturation readouts were outputted, displayed on the monitor and saved. (B) One pulse-oximeter during inflation displaying a foot pulse of 0 bpm and 0 $Sp0_2$ (oxygen saturation). (C) Programmable cuff pump during inflation displays that cuffs were inflated to a pressure of 3.26 psi, with 42 seconds left of inflation (PH, "pressure high") and 2 more cycles left (T#).

2.3.1 Automation of Pulse Oximeter Data Collection

Nonin Medical, the manufacturer of the pulse oximeter was contacted to request the protocol used to output the biometric data over a serial computer connection. The three devices were connected up to a Raspberry Pi 3 (Model B V1.2) via USB. Using the protocol information, a program was written that concurrently read data from the three heart rate monitors and readouts logged as a text file (See **Appendix A** for the written script used).

2.3.2 Automatic Ischemic Conditioning Cuff Pump

The programmable cuff pump was made in-house (**Figure 6**). Briefly, the pump case (8" X 10" X 5", HM3224-ND, DigiKey, Hammond Manufacturing, ON, Canada) housed a pressure gauge, pressure regulator dial, visual display, control dial, 5 hose panel mounts and power supply. Inside the pump enclosure is a vacuum pump (DVH130-V10400X1-0002, HD Mounts). To connect the compressed air to 5 external cuffs, two 2-way air valves (DC 12v, 43237-2, Yosoo) and a 6-outlet manifold (AO-31521-13, Cole-Palmer, IL, USA) were assembled inside the case with 5 triple hose barb panel mounts (PBH-18A-BN, Industrial Specialties Mfg. & IS MED Specialties, CO, USA) on the outside. Five separate cuffs could then be connected to the hose barb mounts with 5 hoses and 5 barbed tube adaptors (48720247, MSC). Once plugged into a power supply (12v, 3amp), the desired pressure applied to all of the cuffs was set using a dial connected to a miniature pressure regulator (MPR1-0, 0-5psi, Dwyer Instruments, IN, USA) and viewed by a pressure gauge (5psi, KC25-5#, Kodiak Controls, IL, USA). The dial of the pressure regulator was attached to the exterior of the box with a panel mounting nut (MPR-N, Dwyer Instruments, IN, USA). The actual pressure produced by the system was measured using a pressure sensor (7psi, MPX5050DP-ND, NXP, USA). This actual pressure could be viewed on a visual display (HD44780, 1602 character, 16X2 LCD, Hitachi). The activation and deactivation of the pump was controlled using a programmable module (Arduino Nano) and an input/output (IO) circuit board which interfaced with the display, valves, sensor and the rotary control dial (See **Appendix B** for the script used). Using the rotary control dial, the number of seconds at high pressure ("PH" = inflation time), number of seconds at low pressure ("PL" = deflation time) and the number of trials ("T#" = number of cycles) could be pre-set and automatically cycled.

Figure 6. Automatic ischemic conditioning cuff pump. (A) Front view with labels and dimensions. Rotary control dial controls the inflation time in seconds (“PH” = pressure high), deflation time in seconds (“PL” = pressure low) and number of cycles (“T#” = trial number). Pressure regulator dial controls the pressure gauge. Actual pressure generated is displayed in the visual display (“P” = pressure in psi). Current settings: 30 seconds remaining of inflation, 2 cycles remaining and 3.25 psi cuff pressure. (B) Back view displaying the 5 inflatable cuffs attached to the pump with labeled parts.

2.3.3 Laser Doppler Imaging

The interruption of blood flow in the hindlimb caused by RIC was confirmed using laser doppler imaging (LDI). The moorLD12 laser Doppler imager (Motor Instruments) with an integrated CCD camera visualizes dermal blood flow of small blood vessels. A combined infrared measurement beam and visible red targeting light beam scanned the shaved skin on the lower leg of a rat (below the cuff) before, during and after cuff inflation. LDI images were collected using the moorLDI^{TM} V5.3 software and displayed as heatmaps with hotter colors indicating higher blood flow.

2.4 MRI and Infarct Analysis

Rats were scanned 24 hours following stroke to obtain T2-weighted magnetic resonance imaging (MRI) images using a 7T General Electric/Agilent RM901 machine. Rats were briefly

anesthetized with isoflurane (4% induction and 2% maintenance). Vital signs were monitored during the 15-minute procedure with a SA Instruments Inc. (SAII) physiological monitoring system. To locate the brain in the MRI, a localizer scan was performed followed by a T2 weighted image of 22 coronal slices, 800 microns thick and a scan time of 8 minutes.

Infarct volumes were estimated by tracing the infarct area using ImageJ software (National Institute of Health, Bethesda MD). All brain images were imported and orientated in ImageJ. All infarct volumes were measured by two trained experimenters who were blinded from group. Each coronal brain section was traced, and the area of infarct from both experimenters were superimposed on to each other and only the overlapping infarct area termed combined infarct area was used. Total brain infarct volumes were estimated by multiplying the sum of combined coronal infarct area measured from slices and multiplied by the slice thickness (eg. 800μm).

2.5 Behavioural Testing

2.4.1 Montoya Staircase Test

The Montoya staircase test assesses skilled forelimb reaching (Montoya, Campbell-Hope, Pemberton, & Dunnett, 1991). Each rat was placed in an enclosed box with two 7-tiered staircases on each side. Three food pellets (45mg Test Diet; Purified Rodent Tablet; 5TUL) were placed on each tier for a total of 21 pellets per side. In the dark, rats had 15 minutes to reach the pellets and the number of remaining pellets was recorded to calculate the amount consumed. Two trials were done per day, 4 hours apart. Animals were trained daily for two weeks to attain a minimum performance of 15 ± 2 pellets from at least one limb. The average of the last 4 trials (2 days) was used for baseline forelimb performance and determined the dominant limb. Testing periods consist of 3 days (6 trials). The first 2 trials allowed the rats to re-familiarize to the task and the last 4 of 6 total trials were averaged to measure the number of pellets retrieved by the

impaired forelimb. Animals were food deprived ~18g/rat during training (daily from week -2 to 0) and testing (for 3 consecutive days at week 0, 1 and 4) to encourage forelimb pellet reaching. Body weight was monitored to not exceed a loss of 5%.

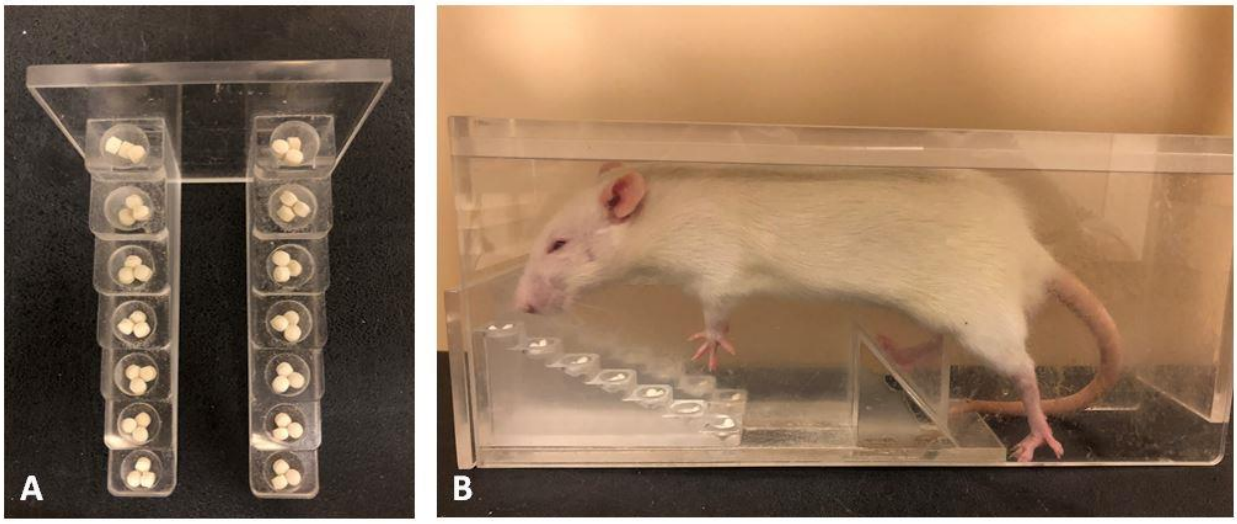

Figure 7. Montoya staircase to measure skilled forelimb reaching. (A) A 7-tier staircase filled with 3 pellets per level per side. A maximum of 21 pellets can be reached per side. (B) Rats were placed inside the staircase enclosure for 15 minutes to reach for the pellets. The number of pellets consumed was counted and the staircases were refilled to be repeated 4 hours later for a total of 2 trials per day.

2.4.2 Tapered Beam Test

The tapered beam test assesses the accuracy of forelimb and hindlimb placement while crossing a tapered beam (length:160cm, width:6cm at start, 1.5cm at end) to a dark box containing a food reward (Schallert, Woodlee, & Fleming, 2002). Over the course of one training day, rats were gradually placed further away from the box until the animal could cross the beam four times without stopping. Baseline performance was measured the following day. On test days, four crosses of the beam were filmed under normal lighting. The number of foot faults and total foot placements for each limb was counted over the four trials. The percent error of the impaired limb was calculated as shown below.

$$\%\ error\ of\ impaired\ limb = \frac{Total\ foot\ faults\ of\ impaired\ paw\ over\ 4\ trials}{Total\ steps\ of\ impaired\ paw\ over\ 4\ trials} \times 100$$

Figure 8. Tapered beam to measure balance and forelimb and hindlimb placement.

2.4.3 Cylinder Test

The cylinder test encourages vertical exploration and assesses spontaneous forelimb use (Timothy Schallert, Fleming, Leasure, Tillerson, & Bland, 2000). The animal is placed in a clear Plexiglas open topped cylinder (30cm tall, 20cm diameter) resting on a clear tabletop and filmed from below for 10 minutes. The task was performed in the dark with enough light from a lamp to view the rat. Once the rat reared onto its hindlimbs and touched the cylinder wall 20 independent times, the trial was over. The number of first touches by either the left or right paw or both at once (bilateral) was recorded. The percent use of the impaired paw was calculated shown below. Only one trial was required per test period and no training was required.

$$\%\ use\ impaired\ paw\ = \frac{0.5\ (\#\ bilateral\ touches)\ +\ 1\ (\#\ impaired\ paw\ touches)}{20\ total\ touches} \times 100$$

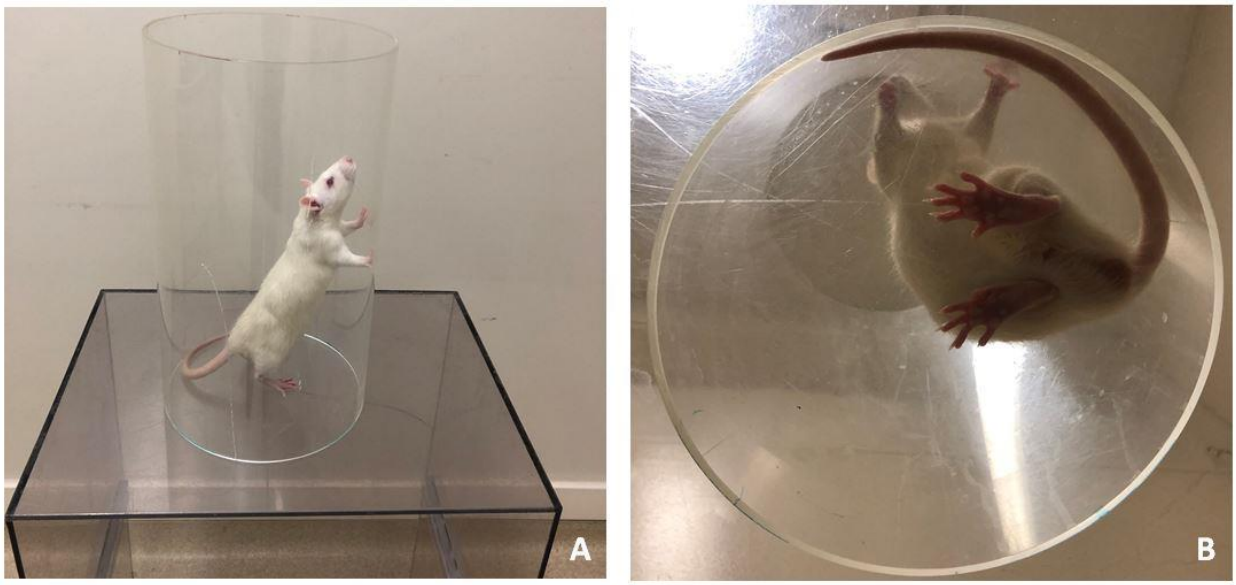

Figure 9. Cylinder to measure spontaneous forelimb use.(A) A rat rearing and exploring. (B) A camera was positioned under the clear tabletop to view and record either left, right or bilateral paw placements.

2.4.4 DigiGait

DigiGait™ is an enclosed motorized transparent treadmill that allows for the analysis of gait parameters from a high speed camera (147 frames/second) located underneath (Dorman, Krug, Frizelle, Funkenbusch, & Mahowald, 2013). Rats were familiarized by gradually increasing the treadmill speed up to a maximum speed of 21cm/second. At this speed, two recordings of 4 to 10 seconds were taken for each rat during a consistent stride (without touching the front or back bumpers). Using the DigiGait™ Video Imaging Acquisition software (Mouse Specifics, Inc) a wide-range output of gait parameters can be assessed (eg. stance width, paw area). Not all rats from *Experiment 2* successfully ran on the treadmill at the required maximum speed, therefore only 16 and 14 rats were analyzed from the control and chronic-RIC groups, respectively. Six "sham" age-matched Sprague-Dawley rats that did not receive a stroke or RIC treatment was used as a reference for "typical" gait measurement outcomes.

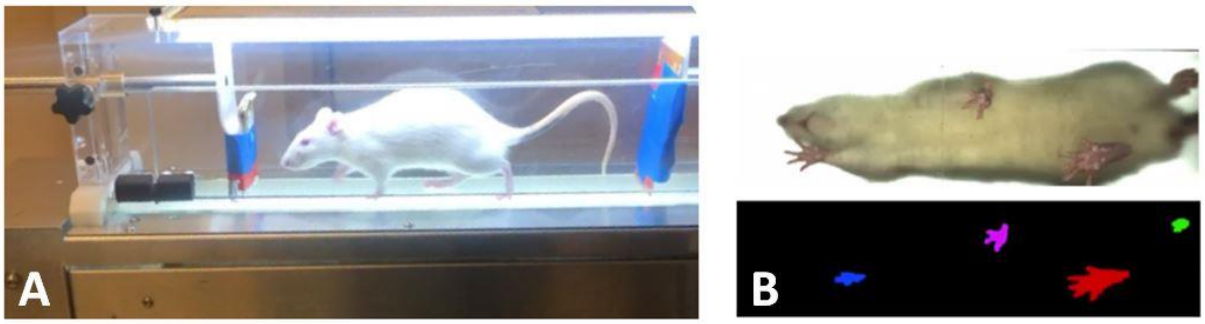

Figure 10. Digigait to assess gait. (A) Rats ran on the DigiGait transparent treadmill and filmed from below. (B) Camera view of DigiGait from below and the detection of paw placements on the treadmill.

2.6 Euthanization

Euthanasia was performed 48 hours after last RIC by administering a lethal dose of euthanyl (65 mg/ml; 2.3 ml/kg) and decapitation.

2.8 Statistical Analysis

Statistical analysis was performed using SPSS Statistics (version 24, IBM Corporation, Armonk, N.Y., USA). To separate large and small stroke sizes, a hierarchal cluster analysis using Ward's Method was performed for all infarct volumes from *Experiment 1*, then again separately for the infarct volumes in *Experiment 2*. This method statistically forms two groups (small and large strokes) ensuring that there is the smallest within-group variation and largest between-group variation. Estimated marginal means were determined for all data. A 4-way ANOVA (group X experiment X sex X stroke size) was used to analyze infarct volumes in *Experiment 1*. Effect sizes (d) of all significant interactions were calculated by Cohen's d using estimated marginal mean and standard deviation. To assess infarct volumes in *Experiment 2*, a 2-way ANOVA (group X stroke size) was used. A 2-way repeated measure ANOVA (group X stroke size) was used to analyze staircase, beam and cylinder in *Experiment 2*. Digigait measured at a single time point in *Experiment 2* was analyzed using 2-way ANOVA (group X stroke size). Post hoc

analysis consisted of multiple comparisons T-tests corrected using the Sidak method. Digigait was analyzed with the Ryan-Einot-Gabriel-Welsch (REGW-F) homogenous subset post-hoc. Significance was set to $p \leq 0.05$. All values are reported as estimated marginal means ±SEM. One rat died in *Experiment 2* but otherwise no rats were excluded from all statistical analyses.

RESULTS

3.1 Efficacy of Remote Ischemic Conditioning (RIC)

Since RIC had previously never been performed in the lab, it was first necessary to test the efficacy of RIC using the developed programmable cuff pump and the cuff method to obstruct blood flow in the hindlimb. Using LDI to detect blood flow of the limb below the cuff, it was established that there was complete ischemia of the limb (indicated by solid blue on LDI) during cuff inflation and reperfusion of blood flow (indicated by red and colours on LDI) during cuff deflation **(Figure 11a)**. In addition, paired with the ischemia during inflation, the limb below the cuff changed colour from pink to blue indicating cyanosis from a lack of oxygen supply (**Figure 11a**). Next, two pulse oximeters (one on each footpad of the rat) was used to compare the pulse and oxygen saturation of a RIC and control limb, each with and without a cuff for an entire RIC session (4 cycles, 5-minute inflation/deflations). Occlusion of the RIC limb was persistent. Cuff inflation caused the foot pulse to drop to 0bpm (from ~350bpm) (**Figure 11b**) and oxygen saturation to drop to 0% (from ~100%) (**Figure 11c**). During cuff deflation, levels would quickly rise back to baseline levels. Readouts from the control limb remained consistent with minimal fluctuations (**Figure 11b, c**). These results emphasize that the RIC method was robust and ready to be used for *Experiment 1* and *2.* During these experiments, the pulse oximeters with data collection was continued to be used to monitor rats during RIC.

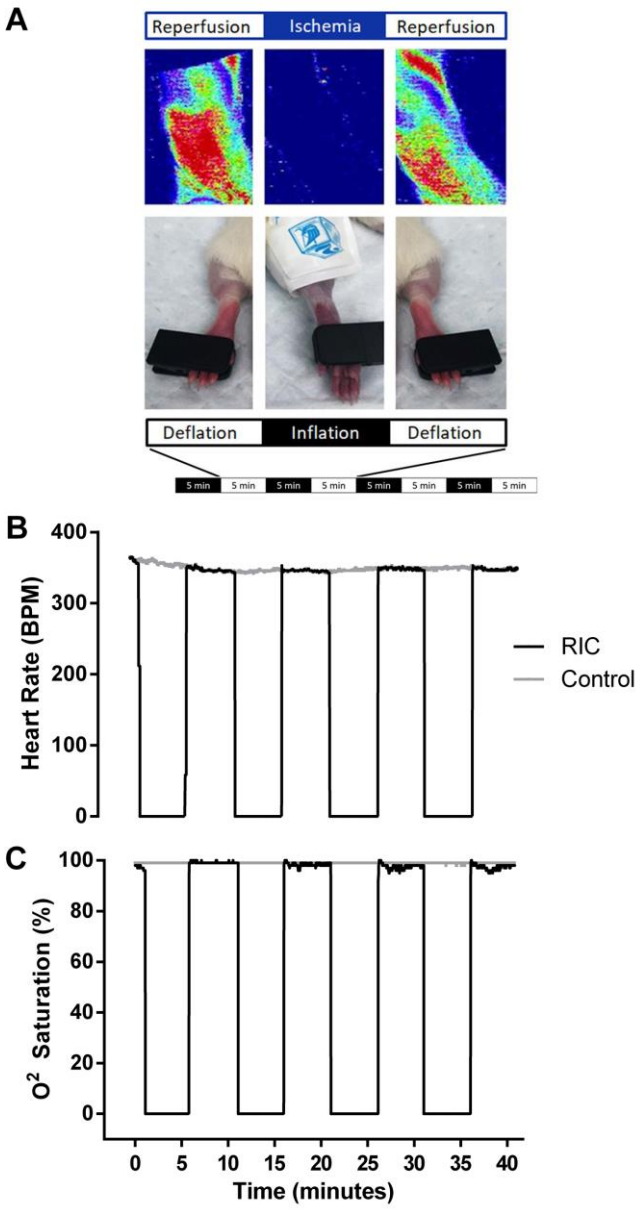

Figure 11. Ischemia of rat hindlimb during remote ischemic conditioning (RIC). (A) Laser doppler imaging (LDI) and limb colour before, during and after cuff inflation. Red indicates high blood flow and blue shows low blood flow with LDI. (B) Pulse and (C) oxygen saturation during a complete bout of RIC (4 cycles of 5-minute inflation (black) and 5-minute deflation (white)). Data obtained using two pulse oximeters, one on each limb of the rat. RIC limb (black) and control non-RIC limb (gray). Representative plotted data from a single rat recorded using a programmed Raspberry Pi.

3.2 Neuroprotection from Remote Ischemic Conditioning (RIC)

The ability of RIC to offer neuroprotection from stroke was quantified by brain infarct volume measured 24 hours after stroke in *Experiment 1*. To assess the efficacy of RIC on different sized lesions, stroke sizes were grouped into 2 hierarchal clusters (small and large strokes) using Ward's method (see Figure 12a for representative images of stroke size). RIC significantly reduced the infarct volume of both small ($p=0.010$) and large ($p<0.0001$) strokes compared to control **(Figure 12b)**. Percent infarct volume reduction was greater with small strokes, at 39% (from 42.4 ± 4.4 to 25.7 ± 4.6 mm^3) compared to 35% (from 146.0 ± 8.3 to 94.5 ± 5.7 mm^3) with large strokes, however the effect was greater in large ($d=1.41$) than small ($d=0.29$) strokes (**Figure 12a, b**). RIC significantly reduced infarct volume in both RIpreC (18 hours before stroke, $p<0.0001$) and RIpostC (4 hours after stroke, $p=0.009$). Pre-conditioning had a stronger neuroprotective effect, reducing infarct volume by 41% (from 116.8 ± 7.4 to 69.0 ± 5.1 mm^3, $d=0.92$) compared to 29% (from 71.6 ± 5.7 to 50.8 ± 5.2 mm^3, $d=0.43$) in post-conditioning (**Figure 12c**). However, infarct volumes were overall larger in RIpreC compared to RIpostC for both RIC ($p=0.014$) and control ($p<0.0001$) groups (**Figure 12c**). Further investigation revealed that infarct volume of RIpreC is only significantly greater than RIpostC within large ($p<0.0001$) but not small strokes ($p=.595$) (**Appendix C Supp. Figure 18**). Both males ($p<0.0001$) and females ($p=0.013$) demonstrated significant neuroprotection from RIC. However, the effect was greater in males ($d=0.94$) and with a greater reduction of infarct volume of 46% (from 102.8 ± 7.0 to 55.8 ± 4.9 mm^3) compared to 23% in females (from 85.6 ± 6.3 to 64.3 ± 5.4 mm^3, $d=0.42$) (**Figure 12d**). Infarct sizes of control males and female rats were not significantly different ($p=0.071$) or between RIC males and females ($p=0.250$).

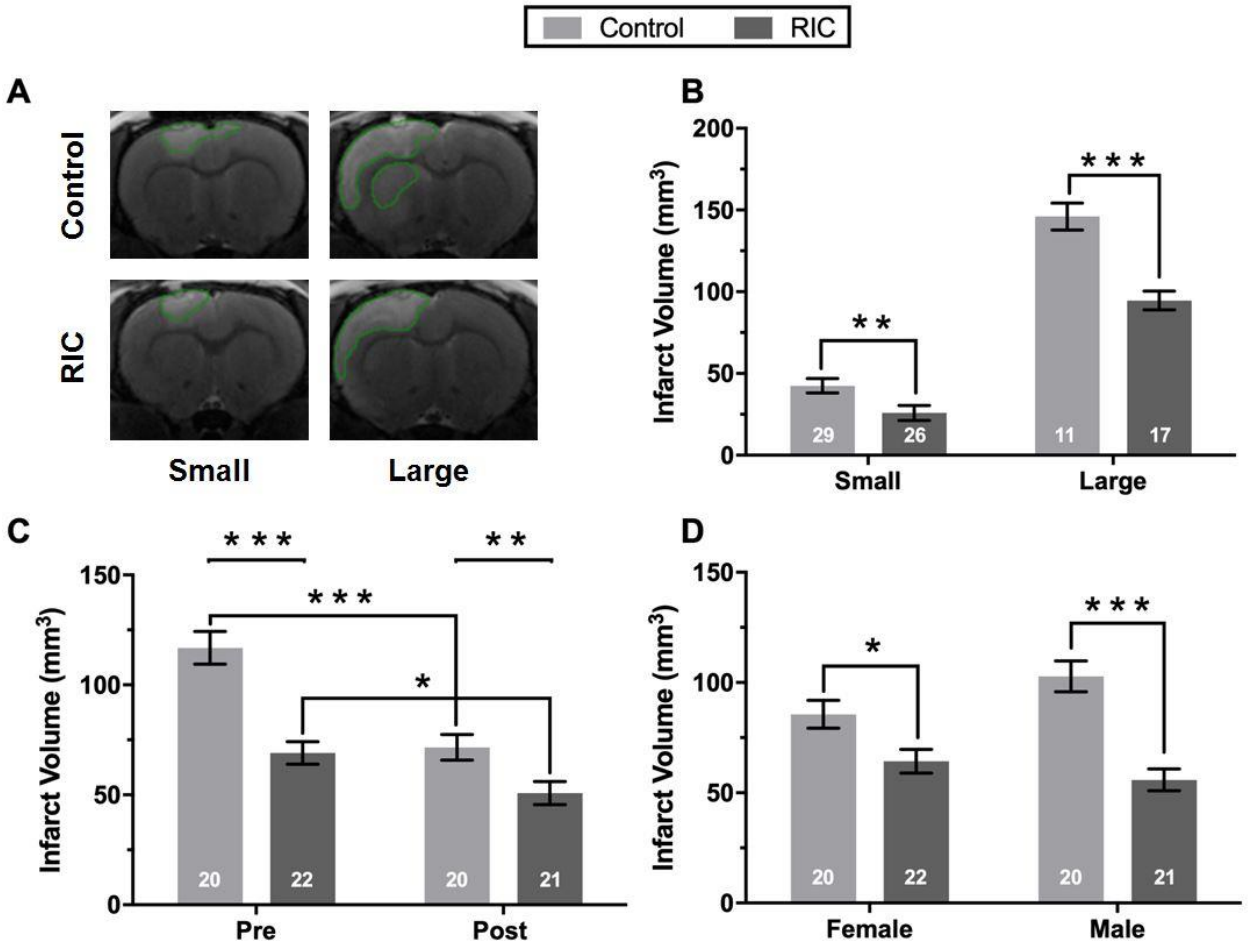

Figure 12. Neuroprotection from RIC. (A) Representative T2-weighted MRI images of small and large strokes in RIC and control groups. Images are all from the same rostral-caudal coronal brain slice. Green indicates the infarcted tissue identified by two independent experimenters. RIC significantly reduces infarct volume in (B) large and small strokes, (C) pre-conditioning (18hr before stroke) and post-conditioning (4hr after stroke), and (D) females and males compared to controls. (C) Post-conditioning animals had significantly smaller stroke volumes than pre-conditioning animals in both RIC and control groups. Number of animals per group is indicated in white (RIC, dark grey; control, light grey). Values are estimated marginal means ± SEM. (*p ≤ 0.05, **p≤ 0.01, ***p≤0.001).

3.3 Restorative Potential of Chronic Remote Ischemic Conditioning (C-RIpostC)

In *Experiment 2*, the potential of chronic-RIC delivered beyond 48 hours, outside the neuroprotective window to promote motor stroke recovery of the affected limb in females was assessed. Although females displayed a more subtle RIC effect than males (**Figure 12d**), females were used in the chronic-RIC study as their smaller body size allowed long-term testing in the

Montoya staircases whereas males become too large to easily fit the chambers after several months. Prior to initiating daily RIC at day 5 post-stroke, infarct volumes were measured 24 hours post-stroke to ensure both experimental groups had equivalent injury based on stroke volume (**Figure 13a**). Similar to *Experiment 1*, stroke sizes were grouped into 2 hierarchal clusters (small and large strokes) using Ward's method. Furthermore, both experimental groups had the same size of large and small strokes (**Figure 13b**). These data confirm that any potential effects of chronic-RIC on motor recovery post-stroke are not due to baseline differences in stroke size.

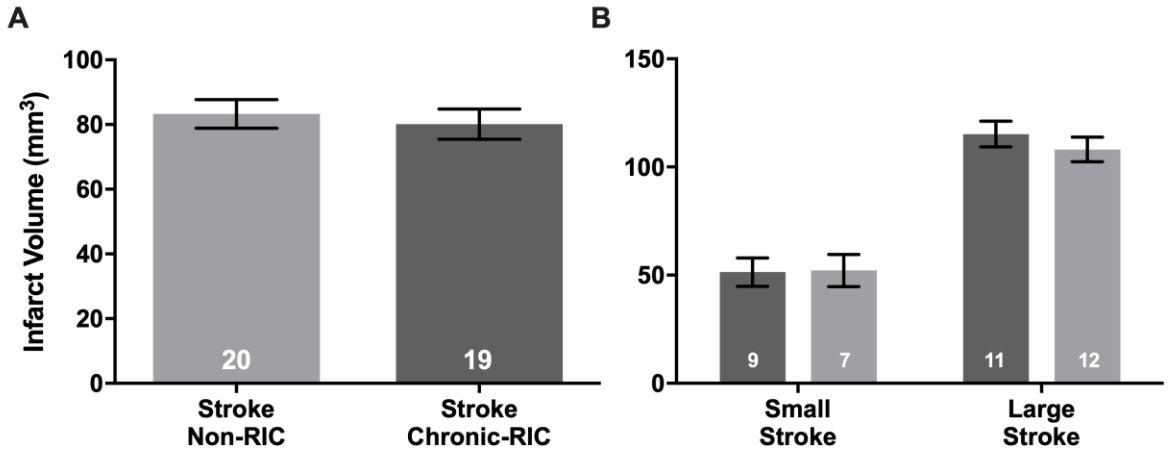

Figure 13. Infarct volumes prior to delivery of chronic RIC. (A) Infarct volumes between the non-RIC and chronic-RIC groups were nearly identical. (B) Both chronic-RIC and control groups had a similar distribution of stroke size. Number of animals is indicated in white. (RIC, dark grey; control, light grey) Values are estimated marginal means ± SEM.

There was no group effect of chronic-RIC on the staircase task ($p=0.932$). However, there was an effect of time ($p<0.0001$) in both groups demonstrating post-stroke impairments on staircase performance 5 days post-stroke (baseline: 17.2 ± 0.3 pellets; Day 5 post-stroke:10.5 ± 0.7 pellets). In addition, rats showed some spontaneous recovery on the staircase task at 12 days post-stroke (Day 5 post-stroke:10.5 ± 0.7 pellets; 12 days post-stroke: 12.2 ± 0.7 pellets) ($p<0.0001$, **Figure 14a**). Rats with small strokes had less impairment than large strokes on the

Montoya staircase beginning day 5 following stroke and onwards ($p<0.01$) (**Figure 14b**). Similarly, in the cylinder task, there was no effect of chronic-RIC ($p=0.667$) but both groups showed a time effect ($p<0.0001$), with a persistent reduction of affected forelimb use from 57.4 ± 3.1% at baseline to 29.2 ± 2.8% at day 5, 31.6 ± 3.3% at day 12, 36.5 ± 3.6% at day 33 (**Figure 15a**). However, rats with small strokes day 12 post-stroke did show some spontaneous recovery, increasing use of the affected forelimb compared to rats with large strokes ($p = 0.017$, **Figure 15b**). On the tapered beam task there was also no group effect in the forelimb ($p=0.919$) or hindlimb ($p=0.572$) errors. However, there was a time effect on post stroke impairment in the affected forelimb ($p<.0001$) and hindlimb ($p<0.0001$). Foot fault frequency increased from baseline to day 5 post-stroke in the forelimb (1.0 ± 0.5 to 8.4 ± 1.2% ($p<0.0001$, **Figure 16a**) and hindlimb (2.1 ± 0.4 to 15.1 ± 1.7%) ($p< 0.0001$, **Figure 16c**). Stroke size significantly influenced hindlimb but not forelimb performance (**Figure 16b**), as hindlimb foot faults were reduced in small strokes post-stroke compared to large ($p<0.05$, **Figure 16d**).

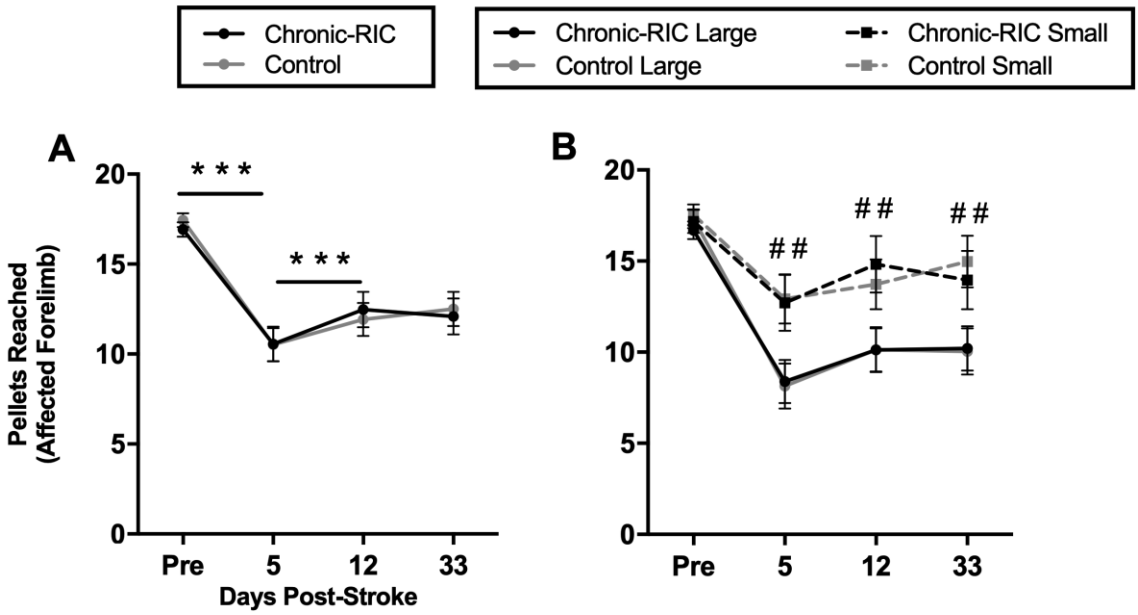

Figure 14. Skilled forelimb reaching in the staircase after stroke and chronic-RIC treatment. (A) Both chronic-RIC (black) and control (gray) groups reached for fewer pellets from pre-stroke (day -3) to the first post-stroke test day (day 5). Both groups also significantly improved (from day 5 to 12). (B) Small strokes (dotted lines) were less impaired on the staircase task than large strokes (solid lines). Values are estimated marginal means ± SEM. (***$p<0.001$, and indicates a time effect, ##$p<0.01$, and indicates the comparison of all small versus large strokes, independent of RIC or not.).

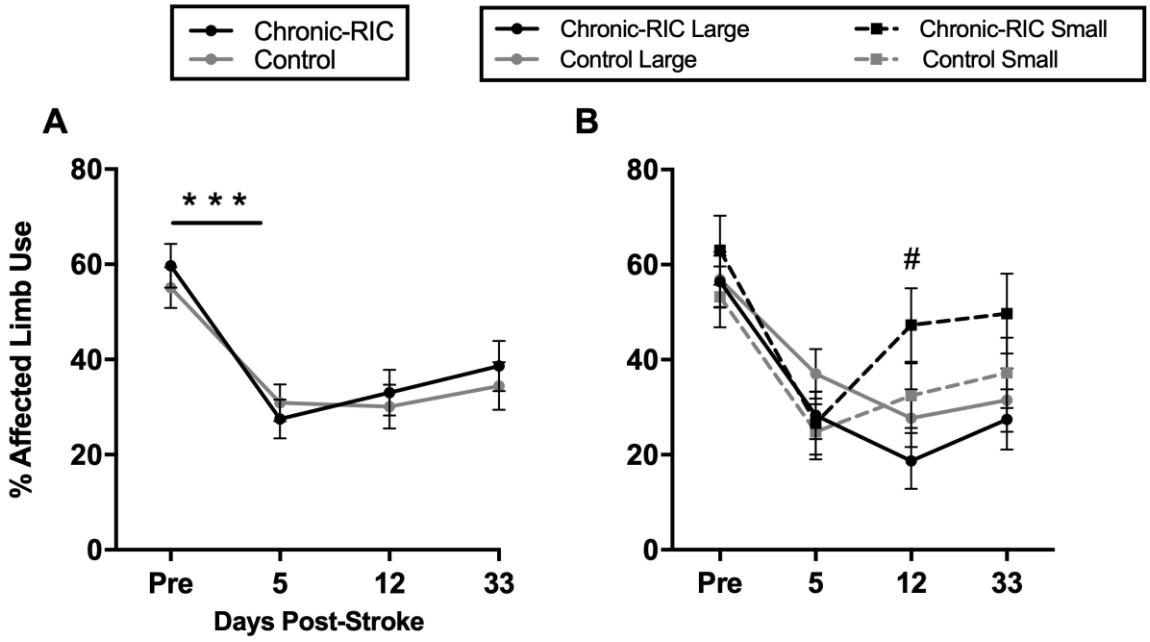

Figure 15. Spontaneous forelimb use in the cylinder task after stroke and chronic-RIC treatment. (A) Both chronic-RIC (black) and control (gray) groups had reduced spontaneous use of affected forelimb from pre-stroke (day -3) to the first post-stroke test day (day 5) and persisted until the last post-stroke test day (day 33). (B) Small strokes (dotted lines) had significantly more spontaneous use of their impaired forepaw 12 days post-stoke than large strokes (solid lines). Values are estimated marginal means ± SEM. (***$p<0.001$, and indicates a time effect; #$p<0.05$, and indicates the comparison of all small versus large strokes, independent of RIC or not)

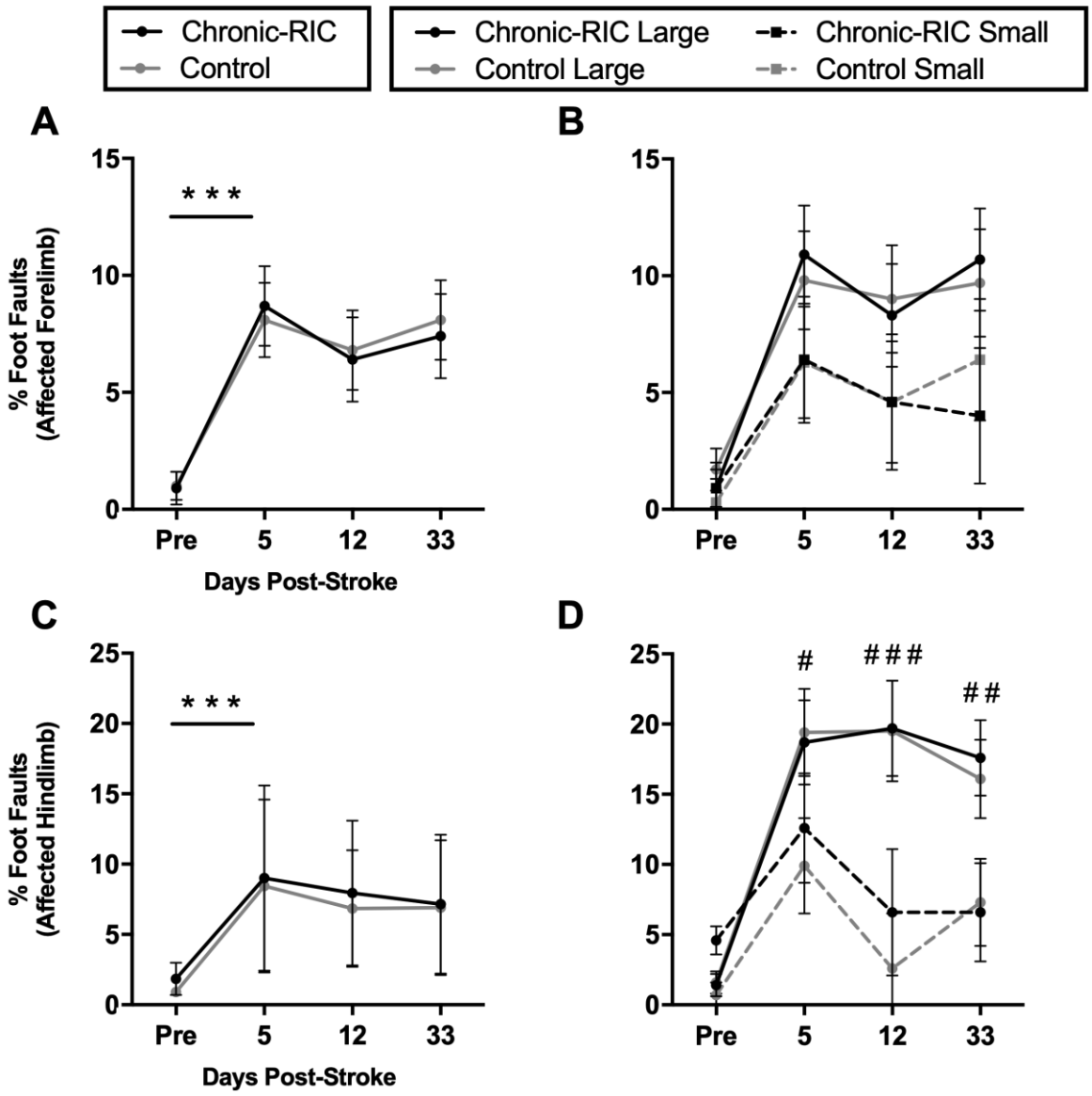

Figure 16. Forelimb and hindlimb placement on the tapered beam after stroke and chronic- RIC treatment. Both chronic-RIC (black) and control (gray) groups had significantly more foot faults from pre-stroke (day -3) to the first post-stroke test day (day 5) in the affected (A) forelimb and (C) hindlimb that lasted until the final post-stroke test day (day 33). (B) Stroke size did not significantly affect forelimb foot faults. (D) Animals with small strokes (dotted lines) had significantly less hindlimb foot faults than large stroke animals at day 5, 12 and 33 post-stroke (solid lines). (***$p<0.0001$, and indicates a time effect; #$p<0.05$, ##$p<0.01$, ###$p<0.001$, and indicates the comparison of all small versus large strokes, independent of RIC or not). Values are estimated marginal means ± SEM.

Using homogenate subsets analysis in panels A, C and E in Figure 17, differences between groups on gait parameters were assessed by identifying groups that were the same. The

variability in stride length (distance a paw travels through a stride) of the affected side measured as standard deviation was significantly greater in shams compared to rats that did not receive chronic-RIC (p=0.010). Although not significantly different than stroke non-RIC rats, rats given chronic-RIC had a stride length variability similar to sham rats (**Figure 17a**). Within large strokes, chronic-RIC significantly increases stride length variability (closer to shams) compared to non-RIC controls (p=0.0014) but there was no difference with small strokes (p=0.704) **Figure 17b**). The swing to stance ratio (time in swing phase to stance phase) of the affected side also showed a similar trend of non-RIC rats having a lower swing to stance ratio (spending more time in stance) (p=0.062) compared to shams. Again, rats that received chronic-RIC appear more similar to sham rats (**Figure 17c**) with a trend of chronic-RIC increasing the swing to stance ratio (more time in swing, closer to shams) compared to non-RIC controls (p=0.061, **Figure 17d**). Lastly, the maximum rate of change of the affected paw during propulsion (eg. how quickly the limb is unloaded from being in stance) was the greatest in chronic-RIC group compared to non-RIC and sham groups (p=0.002, **Figure 17e**). Interestingly, the rate of propulsion was affected by stroke size, being greater in small versus large strokes (independent of RIC treatment or not) but not in the direction of shams (p=0.031, **Figure 17f**).

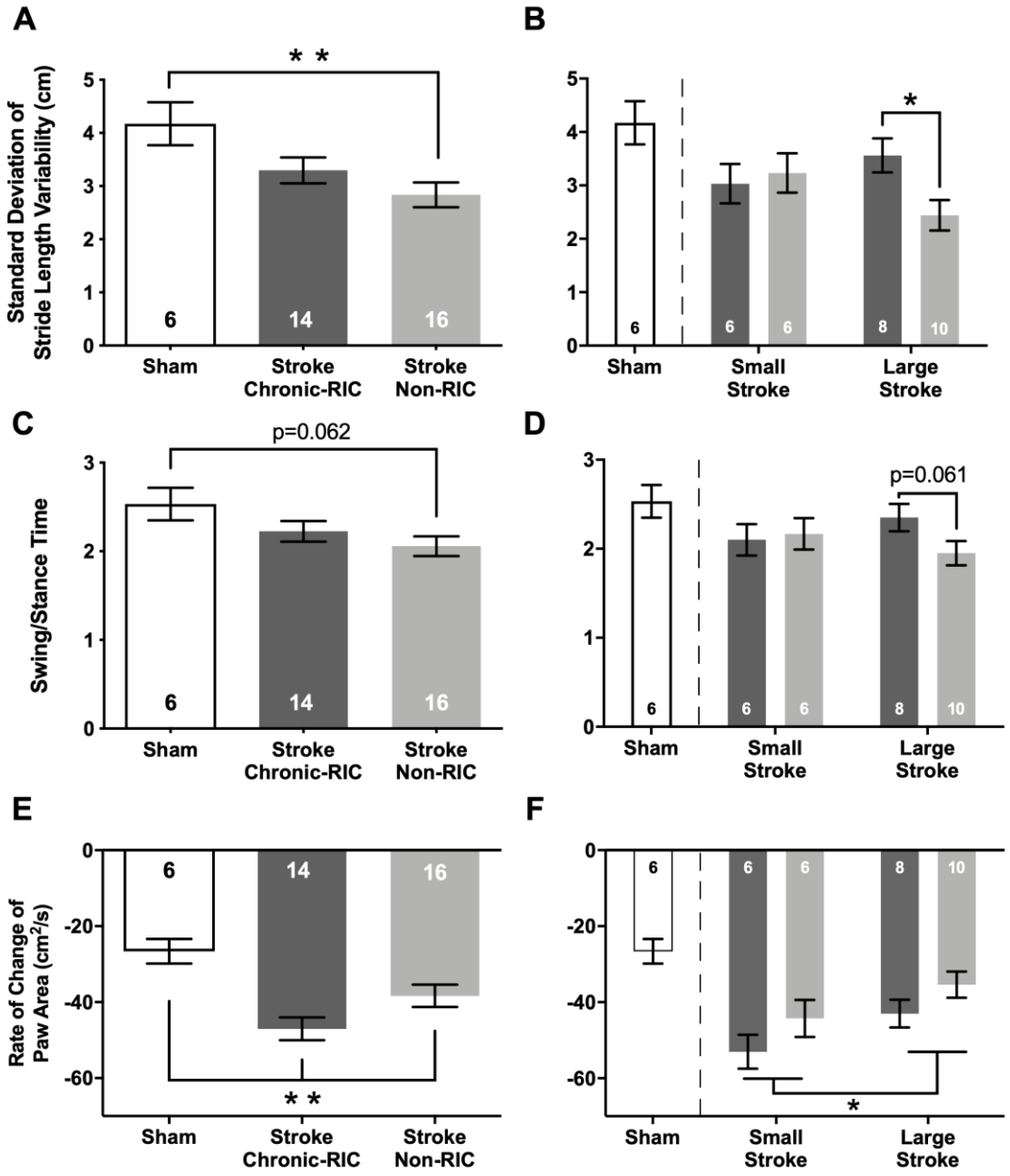

Figure 17. Gait analysis post-stroke and after chronic-RIC. (A) The standard deviation of stride length of the affected side of the body is significantly lower in stroke non-RIC rats compared to sham. (B) RIC significantly increases the standard deviation of stride length within large strokes. (C) The swing to stance time of the affected forelimb trends to being lowest in stroke non-RIC rats compared to sham. (D) There is a trend that RIC increases swing to stance time in large strokes. (E) The rate at which the paw area changes during propulsion is significantly different in all groups with RIC being fastest and sham the slowest. (F) Rats with small strokes have faster rates of propulsion than rats with large strokes. Sham animals represent a "typical" rat gait without any interventions (did not receive a stroke or RIC). Number of animals per group is indicated in white. Values are estimated marginal means ± SEM. (*$p<0.05$, **$p<0.01$).

DISCUSSION

Experiment 1:

There is growing interest in remote ischemic conditioning as a potential therapy for stroke. However, international stroke research guidelines (STAIR and SRRR) stress the importance of pre-clinical work to thoroughly assess the efficacy of potential therapies (eg. in a variety of conditions) prior to clinical translation. This study sought to assess the neuroprotection of RIC using (1) a different pre-clinical stroke model (ET-1) then what had previously been used (MCAO), (2) be the first to compare the effects in males and females within the same study and (3) to deliver RIC at times feasible for patients that is essential to properly evaluate translation potential. Neuroprotection was measured by differences in infarct volume measured 24 hours after ET-1 stroke in males and females with RIpreC (18 hours before) or RIpostC (4 hours after). Our results indicate that all forms of RIC are effective at reducing lesion volume following stroke, however the effects vary based on stroke size, timing of RIC and sex. Neuroprotection from RIC is most effective as pre-conditioning in males with large strokes.

RIC is neuroprotective in the ET-1 stroke model

The exact mechanisms of how RIC exerts its neuroprotective effects are uncertain. However, it was predicted that RIC would be effective in other stroke models (eg. ET-1) based on the evidence that RIC likely functions through multiple pathways (vascular, neural, immunological) (Zhou et al., 2018), exerting benefits at multiple rather than a single target. This hypothesis was supported as RIC reduced infarct volume by a minimum of 23% and maximum of 46% relative to control. These are similar reductions to those previously reported in rats using the MCAO model (**Table 1 and 2)**. Importantly, for the first-time reproducibility of RIC neuroprotection has been demonstrated across stroke models, a recommendation of both STAIR and SRRR.

Although both MCAO and ET-1 model *transient* ischemic stroke, the techniques differ with typically different reperfusion patterns (Windle et al., 2006), injury sizes and locations (Karthikeyan et al. 2018; Liu et al. 2009). Variability within and between stroke models is beneficial to reflect the heterogeneity of stroke in humans. For instance, based on the techniques, filament removal in the MCA during MCAO may closely resemble mechanical clot removal of EVT treatment (K. Lin et al., 2018). Comparatively, the natural gradual reperfusion of ET-1 may resemble spontaneous reperfusion (Gomis & Davalos 2014; Windle et al., 2006). RIC appears successful in both acute scenarios. However, as more stroke patients meet the therapeutic window for tPA, efficacy of tPA with RIC treatment will need to be thoroughly addressed. To date, Hoda and colleagues have shown additive effects of combined tPA and RIC doubling infarct reduction to 50% in an emboli mouse model (Hoda et al., 2012). Altogether, showing efficacy of RIC in an additional stroke models strengthens the likelihood that it will be an effective therapy in stroke patients.

RIC is neuroprotective against clinically relevant large and small strokes

The MCAO stroke model often induces very large injuries (Liu et al., 2009) not representative of human strokes (Edwardson et al., 2017) which is problematic for clinical translation. Despite this, MCAO has been used in all of the RIC neuroprotection studies to date (**Table 1 and 2**). In the rat RIC studies that report infarct volumes in mm^3 (rather than % hemisphere), infarct volume was most often measured 1-day post-stroke with TTC staining resulting in infarcts of 230-380 mm^3 in control that were reduced to 94-250 mm^3 by a single bout of RIC **(Table 1**) (Liu et al., 2016; Hahn et al., 2011; Hu et al., 2012; Bonova & Gottlieb, 2015). Only one study measured infarct volume using MRI (as done in this study) that reported smaller stroke injuries of 146 ± 12 mm^3 in control animals that were reduced to 81 ± 10 mm^3 with RIC (**Table 2**)

(Liang et al., 2018). However, this was following repetitive RIC for 21 days post-stroke, therefore it cannot be compared to infarct volumes from other MCAO studies or from *Experiment 1* that used a single RIC session measured 1-day post-stroke.

One advantage of using the ET-1 stroke model is that it can produce injuries of variable size. Using a 2-hierachial cluster analysis, rats (n=83) were classified into either "large" or "small" strokes. In this study the average large strokes 146 ± 8 mm^3 were reduced to 94 ± 6 mm^3 and small strokes 42 ± 4 mm^3 were reduced to 26 ± 5 mm^3 by a single RIC session. Interestingly, RIC was found to significantly reduce all stroke volumes; reducing large and small strokes by 39% and 35%, respectively. Although not apparent from infarct reduction, between the stroke sizes, the effect was greater in large (d=1.41), compared to small strokes (d=0.29). Nonetheless, RIC may be promising for the treatment of smaller microinfarcts (eg. <1mm^3) (Graff-Radford, 2019), responsible for vascular cognitive impairment.

The large strokes herein (146 mm^3) are less severe compared to stroke volumes with MCAO (~230-380 mm^3, average: ~276 mm^3). Comparing the estimated percent hemisphere that these "large" volumes occupy in the rat brain reveals ~34.0% of the hemisphere with MCAO (~276 mm^3) and ~18.0% (146 mm^3) with ET-1 (Jeffers et al. 2018). Likewise, "small" ET-1 strokes (42 ± 4 mm^3) occupy ~5.2% of the hemisphere. This is in agreement with previous studies in our lab using the ET-1 model, where a stroke volume of 49.5 ± 10.0mm^3 was estimated to make up 6.1% of the total hemisphere (Karthikeyan et al., 2019). Overall, ET-1 strokes produced smaller infarcts than MCAO, but importantly these ET-1 infarcts better represent typical injury size of human ischemic strokes which occupy 4.5-14% (28 to 80 mm^3) of the injured hemisphere (Carmichael, 2005). Our small ET-1 strokes fall within the low end of this range and our large strokes are only 4% beyond the human range of hemisphere infarction. This novel finding

greatly increases the translational potential for RIC showing efficacy not only in very large strokes (i.e. ~34.0% already reported in the literature with MCAO), but also from strokes of ~14.0 %, and ~5.2% with ET-1 that are more clinically relevant.

RIC pre-conditioning is more neuroprotective than post-conditioning with delivery at clinically feasible times

Although RIC neuroprotection studies have demonstrated robust reductions in infarct volume (eg. up to 80% in RIpreC (Wei et al. 2012; Ren et al. 2008) and 67% in RIpostC (Ren et al. 2009)), the majority of studies did not consider the clinical feasibility of the timing of RIC delivery which ranges from immediately to 1 hour before or after stroke (**Table 1 and 2**). Only about 15% of stroke patients receive t-PA within its 4.5 hr time window so even fewer would be able to avail of RIC within an hour of stroke (K. Lin et al., 2018).

In this study RIpreC was delivered 18 hours before stroke, this timing is appropriate as a preventive intervention against first ever or against a second stroke. RIpreC reduced infarct volume by 41% (from 116.8 ± 7.4 to 69.0 ± 5.1 mm^3). This outcome is in agreement with a similar study that delivered RIpreC 12 hours before stroke and reduced infarct volume by ~52% (Ren et al., 2008). The somewhat larger effect reported by Ren and colleagues may be due to a shorter time to stroke (12 versus 18 hours), a more direct RIC method (femoral artery clamp versus cuff), or a longer occlusion time (15 versus 5 minutes) (**Table 1**) (Ren et al., 2008). Interestingly, two RIpreC studies have shown neuroprotection from a single RIC session occurring at 2 and 3 days before stroke reducing lesions by ~44% (Ren et al., 2008) and 40% (Chen, Yang, Lu, Guo, & Dou, 2014), respectively. This supports the view that neuroprotection by RIC may extend up to 72 hours (Zhou et al., 2018). This would enhance translation and

clinical uptake as people may only need to receive RIC 2-3 times per week instead of daily to achieve the same stroke protection.

RIpostC in this study was applied 4 hours after stroke. This ensured that RIC delivery was within the later end of the neuroprotective window which patients must meet for tPA treatment (K. Lin et al., 2018). Delivery 6 hours post-stroke was also previously shown to be non-significant (Ren et al. 2009). RIpostC 4 hours post-stroke in this study reduced infarct volume by 29% (from 71.6 ± 5.7 to 50.8 ± 5.2 mm^3). This is a smaller change than RIC delivered 3 hours post-stroke with infarct reductions of 43% (Ren et al., 2009), ~33% and ~34% (Jing Sun et al., 2012). However, similar to RIpreC, our more conservative reduction in infarct volume may be due to an extra hour between stroke and RIC and a more indirect occlusion method (cuff versus femoral artery clamp) (**Table 2**) (Ren et al., 2009; Sun et al., 2012).

Our results suggest both RIpreC and RIpostC have neuroprotective effects reducing infarct volume by 41% (18 hour) versus 29% (4 hour), respectively. However, the effect is greater in RIpreC with an effect of d=0.92 versus 0.43 in RIpostC. This reported difference is similar to work of Ren et al.(2008, 2009) that used the same RIC parameters in RIpreC as RIpostC and at similar timepoints as *Experiment 1*. RIpreC at 12 hours had larger estimated effect of d=1.65 (Ren et al., 2008) compared to d=1.48 for RIpostC at 3 hours (Ren et al., 2009) (**Table 1 and 2**). The larger effects overall may be due smaller sample size per group (n= 6-8) than the present study (n= 20-21), larger infarct volumes which predispose to larger infarct reduction or the use of direct blood flow occlusion (via femoral artery versus cuff) for longer periods (15 versus 5-minute).

Overall, RIpostC may be an effective treatment within hours after stroke, perhaps it could benefit patients who are not eligible for tPA, or be combined with tPA and RIpostC to offer maximal protection. Meanwhile with greater effects of RIpreC, it may have potential as a preventative therapy to protect people at high risk of first stroke (eg. after the sign of a transient ischemic attack (TIA)) or to prevent recurrent stroke.

RIC offers greater neuroprotection in males than females

Since sex differences exist in stroke and exercise physiology, it was of interest to determine if RIC had equal neuroprotective effects in males and females. Females tend to have smaller strokes (Alkayed et al., 1998) and higher CBF (Peltonen et al., 2015; Rodriguez et al., 1988), than males that may contribute to females having smaller strokes than males. Since RIC has been found to increase CBF (Khan et al., 2018), it was hypothesized that RIC neuroprotection would be enhanced in females as they are innately susceptible to increases in CBF. The opposite results were found as infarct volume of females reduced by 23% (from 85.6 ± 6.3 to 64.3 ± 5.4 mm^3) compared to 46% (from 102.8 ± 7.0 to 55.8 ± 4.9 mm^3) in males with RIC. The only two other RIC studies that used female rats, found infarct reductions of 50% (Li et al., 2015) and 42% (Li et al., 2018) respectively. Surprisingly, infarct reductions were more similar to those seen in males in *Experiment 1*. However, with no direct comparison to males, it difficult to interpret the results and these investigators used immediate RIC which offers greater protection than 18 hour RIpreC and 4 hour RIpostC from *Experiment 1*.

The results also show that the RIC effects were twice as large in males than females (d=0.94 versus 0.42). Although not significant, there is a trend (p=0.071) that females have a smaller mean infarct volume (85.6 ± 6.3 mm^3) compared to males (102.8 ± 7.0 mm^3) in non-RIC groups.

This consistent with existing evidence that females tend to have smaller infarcts than males due to hypothesized neuroprotective effects of estrogen (Alkayed et al., 1998). The final infarct volumes after RIC were also not significantly different between males (55.8 ± 4.9 mm^3) and females (64.3 ± 5.4 mm^3). The larger infarct reduction in males of 46% (versus 23% in females) may partially stem from having larger (although not significant) initial stroke lesions with more salvageable tissue.

It was also predicted that RIC effects would benefit females because sex-differences in cell-death cascades (Manwani and McCullough 2011; Liu et al. 2009). Although both apoptosis pathways exist in both sexes, a caspase-dependent pathway predominates in females versus a caspase-independent pathway in males, (Demarest & McCarthy, 2014), Despite that RIC has been shown to improve mitochondrial function and inhibit caspases (Chen et al., 2018; Slagsvold et al., 2014) that are involved in the caspase-dependent pathway, a greater effect of RIC was seen in males. An alternative hypothesis may be that RIC reduces caspase-independent apoptosis common in males. A key mediator of this pathway is apoptosis inducible factor (AIF) that initiates apoptosis as it migrates from the mitochondria to the nucleus. Previously, nuclear AIF was found to be reduced after local ischemic preconditioning in rats (Lin, Chang, Lee, & Huang, 2009). Although the effect of RIC on AIF has not yet been investigated, perhaps this mechanism provides more neuroprotection in males than females. Furthermore, males may benefit more from RIC than females because RIC increases in CBF help maintain blood flow within regions of the penumbra that would otherwise die. Females, however, may already have this protection from higher CBF intrinsically (Peltonen et al., 2015; Rodriguez et al., 1988).

This was the first study to compare RIC neuroprotection in males and females head to head. Notably, males had nearly twice the reduction of infarct volume than females suggesting that

males might benefit the most from RIC. Future studies should carefully consider RIC efficacy in both sexes.

RIC neuroprotection is robust

RIC reductions in infarct volume in this study were repetitively observed. *Experiment 1* was done in four individual waves, each of which showed the same trend of RIC neuroprotection and smaller infarct volumes. Therefore, not only has this study showed neuroprotection between stroke models but also replication within our lab. Collectively, this reproducibility supports the robust effects of RIC. Effect size of RIC neuroprotection was variable, ranging from d=0.29 to 1.41. When comparing effect sizes between RIC conditions, the largest to smallest effects were the following: Large stroke (d=1.41) > males (d=0.94) > RIpreC (d=0.92) > RIPostC (d=0.43) > females (d=0.42) > small stroke (d=0.29). According to Cohen's interpretation, RIC has a large effect (d=0.8) in large strokes, males and RIpreC, a medium effect (d=0.5) in RIpost and females and a small effect (d=0.2) in small strokes (Wassertheil & Cohen, 2006). When comparing to mean effect sizes estimated from the literature, RIpreC and RIpostC have effects sizes of d=3.61 and d=2.22, respectively (**Table 1 and 2**). The effect sizes from *Experiment 1* are in agreement showing that RIpreC (d=0.92) has a greater effect than RIpostC (d=0.43). Nonetheless, the effect sizes from the present study are smaller than those in the literature. One reason is that MCAO studies used fewer rats (average 8 per group) than this study (average 21 per group) to achieve significance and a smaller N is associated with greater variability (SD) that may produce greater effect size. Secondly, since it was determined herein that the largest RIC effects are detected in larger strokes (d=1.41), the effects in the literature may be greater due to the larger strokes induced by MCAO versus ET-1. Finally, in many of the published studies it is unclear how many animals were excluded from the analyses and for what reasons.

Although RIC neuroprotection appears to be robust within days post-stroke, it will be important to determine if the effects persist in the long-term. For instance, Ren and colleagues delivered a bout of RIC immediately after stroke but measured infarct using two methods at two time points (Ren et al. 2009). Two days after stroke, TTC staining revealed a larger infarct reduction (67%) than with cresyl violet staining (24%), perhaps highlighting the potential of TTC to overestimate infarcted tissue (Benedek et al. 2006; Sommer 2016). However, after 2 months there was no reduction in infarct volume with cresyl violet compared to day 2 (0% versus 24%). This suggests neuroprotection benefits may not persist. Interestingly at 3 months post-stoke, Dooley and Corbett (1998) previously demonstrated some precedence for erosion of cerebral ischemic pre-conditioning. However, continuing RIC may mitigate lost neuroprotection as the same protection was found at 1 and 2 weeks post-stroke with repetitive RIpostC (Ren et al., 2015). Nonetheless, RIC neuroprotective effects appear sufficiently robust to be considered as a stroke neuroprotective therapy.

Limitations

This study further assessed the translational potential of RIC and demonstrated efficacy in a second stroke model (ET-1 versus MCAO) and in both sexes. However, there are still remaining factors to consider such as how RIC would translate in aged animals (especially post-menopausal females) or those with existing comorbidities such as hypertension or diabetes. Although this study answered questions of RIC timing (pre versus post), effect on stroke size and sex differences, the optimal RIC parameters (eg. dose) are still unknown. This study used the most common RIC protocol being applied in clinical studies. Given the variable effects of RIC in this study using the same RIC protocol, it may be challenging to detect noticeable differences in

neuroprotection between the two most common protocols of 3 X 10-minute versus 4 X 5-minute sessions, 1 versus 2 limbs or RIC of the arm versus thigh.

Implications of the study

This study has provided valuable and highly translatable information for the application of RIC for stroke neuroprotection and honed in on populations of stroke patients likely to benefit from RIC treatment. Previously, RIC protection in MCAO stroke models showed efficacy in large injuries, occupying approximately 34.0% of the injured hemisphere. For the first time, using an ET-1 stroke model, RIC appears protective against both "small" and "large" strokes of ~5.2% and ~18.0% hemisphere volume, which are more similar to the hemispheric injuries of 4.5-14% found in the clinical population. With significant protection across different stroke sizes, RIC may also offer protection against the microinfarcts that contribute to vascular cognitive impairment. Pre-conditioning (RIpreC) is more effective than post-conditioning (RIpsotC). This suggests that RIC treatment may be very beneficial for preventing or reducing the incidence of recurrent strokes. For instance, people who experience a mini-stroke (TIA) or an ischemic stroke may benefit from daily or even intermittent RIC at home. Within 5 years of ischemic stroke, 20% of survivors have a recurrent stroke (Dhamoon, Sciacca, Rundek, Sacco, & Elkind, 2006) and 23% of ischemic stroke survivors first experience a TIA, often days or weeks before the stroke event (Rothwell & Warlow, 2011). Preventing or reducing the severity of recurrent stroke would not only improve quality of life with less impairments but reduce the burden on caregivers and the healthcare system. Lastly, RIC is twice as effective in males than females, therefore males may benefit more from RIC treatment.

Experiment 2:

Many stroke survivors are sedentary post-stroke (Bernhardt et al., 2004) and develop a reduced capacity for exercise (MacKay-Lyons & Makrides, 2002) compromising potential future rehabilitative gains (Hyngstrom et al., 2018). Despite the knowledge that aerobic exercise is beneficial for recovery post-stroke, many patients do not reap the benefits (Mang et al., 2013). Since RIC can be performed with relative ease compared to exercise and may share similar protective and regenerative pathways (Alcantara et al., 2018; Austin et al., 2014; Khan et al., 2018; Ramagiri & Taliyan, 2017) important for brain repair and recovery after stroke (Corbett et al., 2017), RIC may have potential to enhance stroke recovery. The aim of this study was to begin chronic-RIC (C-RIpostC) within the stroke recovery phase (beyond the window of neuroprotection) to determine if repetitive RIC for 28 days could improve motor recovery following ET-1 stroke. Motor recovery of the impaired limb was assessed on the Montoya staircase, cylinder, tapered beam and Digigait. Overall, behavioural data shows that stroke resulted in motor impairments of the affected limb, however, chronic-RIC did not improve motor recovery.

Chronic-RIC alone does not improve motor recovery following stroke

Although it was predicted that C-RIpostC would improve motor recovery, RIC provided no benefit to motor recovery in the Montoya staircase, cylinder or tapered beam tasks relative to control. Some behavioural tasks showed limited spontaneous recovery that is typical of human stroke (Kwakkel, Kollen, & Twisk, 2006). Specifically, spontaneous recovery was detected in all rats on the Montoya staircase between day 5 and 12 post-stroke as well as day 12 on the cylinder task for rats with small strokes, although not on the tapered beam task. Spontaneous recovery

was most detectable on the Montoya staircase compared to other tasks as it has the highest sensitivity to detect change (Corbett et al., 2017).

After 28 days of C-RIpostC only three gait parameters indicated borderline beneficial effects, one being significant. Among larger strokes, daily RIC significantly increased stride length variability ($p=.014$), closer to the variability exhibited by non-stroke rats. Greater stride length variability in non-stroke animals may signify that they have greater mobility with more possible stride lengths than stroked animals. A similar benefit of C-RIpostC in large strokes was seen with swing to stance time (although only a trend, $p=.061$). A greater swing-stance time in non-stroke rats indicates that the affected limb is elevated longer (swing) instead of being rapidly returned to the ground (stance), suggestive of better stability and balance.

Since RIC can be considered an exercise mimetic, perhaps chronic-RIC of the limb (or exercise) lacks specificity needed to improve skilled forelimb reaching (eg. Montoya staircase) alone and thus may only provide subtle improvements in gait as seen herein (eg. stride length variability and swing-stance time). Numerous pre-clinical interventions such as the delivery of growth factors (Jeffers et al., 2014), environmental enrichment (Biernaskie & Corbett, 2001), reach training and exercise by themselves are effective in promoting stroke recovery only when used in combination with rehabilitation (Clarke, Langdon, & Corbett, 2014; Jeffers & Corbett, 2018). Therefore, the delivery of C-RIpostC with concurrent upper-limb rehabilitation may be needed to achieve benefits for upper limb motor recovery.

The above results are in contrast to findings from another C-RIpostC study that showed motor improvement in beam and ladder tests post MCAO stroke with daily RIC for 21 days (Liang et al., 2018). A serious confound of this study, and a reason for the design of the present study, was

that RIC began 2 days after MCAO stroke, a time potentially within the window of neuroprotection as infarct volume can change with treatments delivered between day 3-5 post-stroke (Carmichael, 2016). In support of this contention, C-RIpostC in this study reduced infarct volume by 44% (to 81 ± 10 mm^3 from 146 ± 12 mm^3 in control) after 21 days of C-RIpostC. Such a large reduction in infarct volume post-stroke is indicative of significant neuroprotection and it is thus highly questionable if functional gains were related to regenerative or restorative mechanisms. In contrast, the present C-RIpostC study began treatment 5 days post ET-1 stroke, which is optimal for beginning stroke recovery treatments and beyond the window of neuroprotection (Sun and Murphy 2010).

Limitations

In this study the effect of chronic-RIC to promote motor stroke recovery was only assessed in females. For long-term behavioural tests, males grow too large for the behavioural apparatus (eg. the Montoya staircase). Therefore, a direct comparison of the effect of chronic-RIC between sexes was not possible. Since RIC neuroprotection was twice as effective in males in *Experiment 1*, it could be assumed that males would also exhibit better possible outcomes from RIC in the recovery phase. However, given that there were no trends of chronic-RIC to improve post-stroke motor recovery (eg. in Montoya staircase, cylinder and tapered beam) it is unlikely that males would experience significantly greater effects. Lastly, this study only looked at the individual effect of chronic-RIC on motor stroke recovery and it is possible that a combination approach with rehabilitative therapy is needed to enhance recovery.

Implications of the study

This study was designed with the goal that RIC may help stroke survivors who are often sedentary (Bernhardt et al., 2004), unmotivated or physically deconditioned to participate in physical activity which is known to be benefit recovery (Mang et al., 2013). Based on the success of RIC as a pre-clinical neuroprotective therapy (*Experiment 1*) and the possible overlaying benefits of RIC and aerobic exercise (Khan et al., 2018), this pre-clinical study aimed to assess the potential of daily RIC (C-RIposC) alone (possibly mimicking exercise) to improve stroke recovery. Although the findings were negative, it may be that RIC in the recovery phase does have strong enough effects to stimulate regenerative and neuroplastic processes to be detected at the behavioural level (eg. skilled reaching or beam walking). However, C-RIpostC may have subtle benefits, existing at the protein level for instance. Therefore, although RIC did not improve motor stroke recovery, there may still be useful applications of C-RIpostC to prime underlying processes necessary for recovery either delivering it before or concurrently with rehabilitation which has shown to be an effective approach previously in the stroke recovery field.

CONCLUSION

The potential of RIC to elicit neuroprotection (to reduce stroke volume) and neurorecovery (to improve motor stroke recovery) was evaluated. One session of RIC delivered at clinically feasible times was neuroprotective using the ET-1 model demonstrating replication between stroke models (with MCAO). ET-1 produced smaller strokes overall ("small":5.2%, "large":18%) than MCAO (~34%) and thus are more relevant to injury sizes seen clinically (4.5-14%). RIC is effective across stroke sizes, although to a greater extent with "large" strokes. Pre-conditioning (RIpreC) at 18 hours was more effective than post-conditioning (RIpostC) at 4

hours, suggesting that RIpreC may be especially beneficial for preventing stroke or reducing the likelihood of recurrent stroke. Interestingly, males appeared to obtain about twice the protective benefits of RIC compared to females. While this study shows promise for possible translation of RIC to the clinic, further pre-clinical research is needed. For example, it is unclear if neuroprotection persists in the long-term and whether the same level of neuroprotection can be attained in aged animals especially those with comorbidities typical of stroke patients. Although daily application of RIC in the recovery phase did not promote motor recovery, RIC may still benefit stroke recovery if delivered in combination with rehabilitation.

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APPENDIX A – Script for Pulse Oximeter Data Collection

```
import serial
from multiprocessing import Process
import time
import binascii
from time import sleep
SAMPLE_RATE = 1
class Packet:
	def __init__(self, data, packetType):
		self.packetType = packetType
		self.data = data
def read_packet(HRSerInterface):
	while(1):
		if(HRSerInterface.read() == b'\xff'):
			keyByte = HRSerInterface.read()
			paramCodeStart = HRSerInterface.read()
			paramVal = HRSerInterface.read(1)
			paramCodeEnd = HRSerInterface.read()
			if(paramCodeStart == b'\xf4'):
				return Packet(str(int.from_bytes(paramVal, byteorder='little')), 4)
			if(paramCodeStart == b'\xf5'):
				return Packet(str(int.from_bytes(paramVal, byteorder='little')), 5)
			if(paramCodeStart == b'\xf6'):
				return Packet(str(int.from_bytes(paramVal, byteorder='little')), 6)
			if(paramCodeStart == b'\xf7'):
				return Packet(str(int.from_bytes(paramVal, byteorder='little')), 7)
def monitor_stream(deviceNum):
	startTime = time.time()
	try:
		hrSerInterface = serial.Serial("/dev/ttyUSB" + str(deviceNum))
	except:
		print("Unable to open /dev/ttyUSB" + str(deviceNum))
		return -1
	try:
		hrCsv = open("hr" + str(deviceNum) + ".csv", 'w')
	except:
		print("Unable to open hr" + str(deviceNum) + ".csv!")
		return -1
	sampleTimer = time.time()
	while(1):
		currentTime = time.time()
		if (currentTime - sampleTimer < SAMPLE_RATE):
			read_packet(hrSerInterface)
```

```
                else:
                        hrHundredsData = None
                        hrHundredsReceived = False
                        hrTensData = None
                        hrTensReceived = False
                        spO2Data = None
                        spO2Received = False
                        plethysmogramData = None
                        plethysmogramReceived = False
                        while(not hrHundredsReceived or not hrTensReceived or not
spO2Received or not plethysmogramReceived):
                                packet = read_packet(hrSerInterface)
                                if (packet.packetType == 4):
                                        hrHundredsData = packet.data
                                        hrHundredsReceived = True
                                elif (packet.packetType == 5):
                                        hrTensData = packet.data
                                        hrTensReceived = True
                                elif (packet.packetType == 6):
                                        spO2Data = packet.data
                                        spO2Received = True
                                elif (packet.packetType == 7):
                                        plethysmogramData = packet.data
                                        plethysmogramReceived = True
                        combinedHR = int(hrHundredsData) * 100
                        combinedHR += int(hrTensData)
                        csvLine = str(combinedHR) + "," + spO2Data + "," + plethysmogramData +
"," + str(currentTime - startTime) + "\n"
                        hrCsv.write(csvLine)
                        print("Monitor" + str(deviceNum) + ": " + csvLine)
                        sampleTimer = time.time()
hr0Proc = Process(target=monitor_stream, args=(0,))
hr1Proc = Process(target=monitor_stream, args=(1,))
hr2Proc = Process(target=monitor_stream, args=(2,))
hr3Proc = Process(target=monitor_stream, args=(3,))
hr0Proc.start()
hr1Proc.start()
hr2Proc.start()
hr3Proc.start()
sleep(10)
```

APPENDIX B – Script for Ischemic Conditioning Cuff Pump

```
//YWROBOT
//Compatible with the Arduino IDE 1.0
//Library version:1.1
#include <Wire.h>
#include <LiquidCrystal_I2C.h>
LiquidCrystal_I2C lcd(0x3f, 20, 4); // set the LCD address to 0x27 for a 16 chars and 2 line display
#include <MD_REncoder.h>
#include <EEPROM.h>
// set up encoder object
MD_REncoder R = MD_REncoder(2, 3);
#define BUTTON 4
#define Psensor A0
boolean bData = false;
int timer1_counter= 34286>>2;   // preload timer 65536-16MHz/256/2Hz;
const byte interruptPinTemp = 6;
uint16_t Pon = 0;
uint16_t Poff = 0;
uint16_t Ptrials = 0;
float    measuredP=0.0;
volatile uint16_t PsenVal;
#define LEDPIN 13
//----------------------------------------
void writeEEprom(int p_address, unsigned int p_value)
{
  p_address <<= 1;
  byte lowByte = ((p_value >> 0) & 0xFF);
  byte highByte = ((p_value >> 8) & 0xFF);
  EEPROM.write(p_address, lowByte);
  EEPROM.write(p_address + 1, highByte);
}
unsigned int readEEprom(int p_address)
{
  p_address <<= 1;
  byte lowByte = EEPROM.read(p_address);
  byte highByte = EEPROM.read(p_address + 1);
  return ((lowByte << 0) & 0xFF) + ((highByte << 8) & 0xFF00);
}
//----------------------------------------
void  retreveEEpromData() {
  Pon = readEEprom(0);
  Poff = readEEprom(1);
  Ptrials = readEEprom(2);
```

```
}
void  initEEpromData() {
  writeEEprom(0, 0);
  writeEEprom(1, 0);
  writeEEprom(2, 0);
}
//============================================================
void  initDisplay() {
  char LOCstr[17];
  lcd.setCursor(0, 0);  //Pressure on
  sprintf(LOCstr, "PH=%.4d", Pon);
  lcd.print(LOCstr);
  lcd.setCursor(0, 1);   //Presure off
  sprintf(LOCstr, "PL=%.4d", Poff);
  lcd.print(LOCstr);
  lcd.setCursor(8, 0);   //Trial#
  sprintf(LOCstr, " T#=%.2d",Ptrials);
  lcd.print(LOCstr);
  lcd.setCursor(8, 1);
  sprintf(LOCstr, " Pr=%d", PsenVal);
  lcd.print(LOCstr);
}
//MPX5050 === Vout = VS (P x 0.018 + 0.04)
//PIN 1.  VOUT
//2.  GROUND
//3.  VCC
//4.  V1
//5.  V2
//6.  VEX
//============================================================
void  DisplayItem(char * head, char * value) {
  lcd.clear();
  lcd.setCursor(0, 0);
  lcd.print(head);
  lcd.setCursor(0, 1);
  lcd.print(value);
}
volatile int cnt = 0;
volatile int cnt1 = 0;
volatile boolean state = false;
volatile boolean oldstate = false;
void beriTemperatura(){
  cnt++;
}
```

```
//////////////////////////Timer interrupt
ISR(TIMER1_OVF_vect)        // interrupt service routine
{
  cnt1 = cnt ;              // copy value to some other variable
   bData = true;            // set a flag
   cnt = 0;  // reset counter value
   TCNT1 = timer1_counter;   // preload timer
   PsenVal= analogRead(Psensor);
 state = !state;  // toggle
 digitalWrite (LEDPIN, state ? HIGH : LOW);
}
void dispPressure()
{
 char LOCstr[17];
 if (state != oldstate){
    lcd.setCursor(12, 1);
    sprintf(LOCstr, "%.2d", PsenVal);
    lcd.print(LOCstr);
    oldstate=state;
 }
}
//---------------------------------
void setup()
{
 Serial.begin(57600);
 /////IRQ code
  noInterrupts();          // disable all interrupts
 pinMode(interruptPinTemp, INPUT_PULLUP);
 attachInterrupt(digitalPinToInterrupt(interruptPinTemp), beriTemperatura, CHANGE);
 TCCR1A = 0;
 TCCR1B = 0;
 //timer1_counter = 34286;   // preload timer 65536-16MHz/256/2Hz
 TCNT1 = timer1_counter;   // preload timer
 TCCR1B |= (1 << CS12);    // 256 prescaler
 TIMSK1 |= (1 << TOIE1);   // enable timer overflow interrupt
 interrupts();           // enable all interrupts
 //////end IRQ code
 pinMode(LEDPIN, OUTPUT);
 digitalWrite(LEDPIN,LOW);
 lcd.init();                  // initialize the lcd
 lcd.init();
 // Print a message to the LCD.
 lcd.backlight();
 lcd.clear();
```

```
  lcd.setCursor(3, 0);
  lcd.print("Hello, world!");
  // initEEpromData();
  delay(1000);
  lcd.clear();
  R.begin();
  pinMode(BUTTON, INPUT_PULLUP);
  retreveEEpromData();
  initDisplay();
}
//--------------------------
uint8_t menuitem = 0;
const uint8_t numMenuItems = 4;
//------------------------------------------------------
void menu() {
  uint8_t x = R.read();
  uint8_t b = digitalRead(BUTTON);
  dispPressure();
  char menustr[17], titlestr[17];
  if (x) {
    menuitem = (menuitem + (x == DIR_CW ? +1 : -1)) % numMenuItems;
    Serial.print (menuitem);
    switch (menuitem)
    {
      case (0): {
          Serial.print (" 0\n");
          sprintf(menustr, "%.4d sec      ", Pon);
          sprintf(titlestr, "PressureON     ");
          DisplayItem(titlestr, menustr);
          break;
        }
      case (1): {
          Serial.print (" 1\n");
          sprintf(menustr, "%.4d sec      ", Poff);
          sprintf(titlestr, "PressureOFF     ");
          DisplayItem(titlestr, menustr);
          break;
        }
      case (2): {
          Serial.print (" 2\n");
          sprintf(menustr, "%.4d          ", Ptrials);
          sprintf(titlestr, "Num of trials   ");
          DisplayItem(titlestr, menustr);
          break;
```

```
    }
   case (3): {
      Serial.print (" 3\n");
      lcd.clear();
      lcd.setCursor(3, 0);
      lcd.print("Go");
      delay(500);
      initDisplay();
      break;
    }
   case (4): { //not currently used
      //Serial.print (" 4\n");
      break;
    }
   default: {
      //Serial.print ("default");

    }
  }//switch
}//if
if (!b) {
  while (!b) {
   dispPressure();
   b = digitalRead(BUTTON);
   delay(50);
  }
  switch (menuitem)
  {
   case (0):
   case (1):
   case (2): {  //get/set new item into eeprom
      int16_t LOCvalue = readEEprom(menuitem);
      char LOCchar[6];
      while (b) {
       x = R.read();
       if (x) {
         LOCvalue = (LOCvalue + (x == DIR_CW ? +1 : -1));
         if (LOCvalue > 1500) LOCvalue = 0;
         if (LOCvalue < 0) LOCvalue = 1500;
         sprintf(LOCchar, "%.4d", LOCvalue);
         lcd.setCursor(0, 1);
         lcd.print(LOCchar);
       }
       //dispPressure();
```

```
      b = digitalRead(BUTTON);
    }
    writeEEprom(menuitem, LOCvalue);
    while (!b) {
      // dispPressure();
      b = digitalRead(BUTTON);
      delay(50);
    }
    retreveEEpromData();
    Serial.print(menuitem); Serial.print("="); Serial.print(LOCvalue);
    break;
  }//END get/set new item into eeprom
case (3): { //GO
    unsigned long Ctime = millis();
    uint16_t LOCtrials = 0;
    char LOCstr[17];
    while (b && (LOCtrials < Ptrials)) { ////////////// Trials
      uint16_t LOCon = 0;
      uint16_t LOCoff = 0;
      while (b && (LOCon < Pon)) {      ////////////// On
        //turn solenoid on
        Ctime = millis();
        while ((Ctime + 1000) > (millis())) {
          dispPressure();
          b = digitalRead(BUTTON);
          if (!b) break;
        }
        LOCon++;
        lcd.setCursor(5, 0);
        sprintf(LOCstr, "%.4d", Pon - LOCon);
        lcd.print(LOCstr);
      }
      if (!b) break;
      Ctime = millis();
      while (b && (LOCoff < Poff)) {      ////////////// off
        //turn solenoid off
        Ctime = millis();
        while ((Ctime + 1000) > (millis())) {
          dispPressure();
          b = digitalRead(BUTTON);
          if (!b) break;
        }
        LOCoff++;
        lcd.setCursor(5, 1);
```

```
          sprintf(LOCstr, "%.4d", Poff - LOCoff);
          lcd.print(LOCstr);
          if (!b) break;
         }
         LOCtrials++;
         lcd.setCursor(12, 1);
         sprintf(LOCstr, "%.2d", Ptrials - LOCtrials);
         lcd.print(LOCstr);
        }
        if (!b) {
         //turn solenoid off
        }
        initDisplay();
        break;
       }//END go
     case (4): { //not currently used
        break;
       }
     default: {
       }
   }//swithch
  }//if
}//menu

//---------------------------
void loop()
{
  menu();
}
```

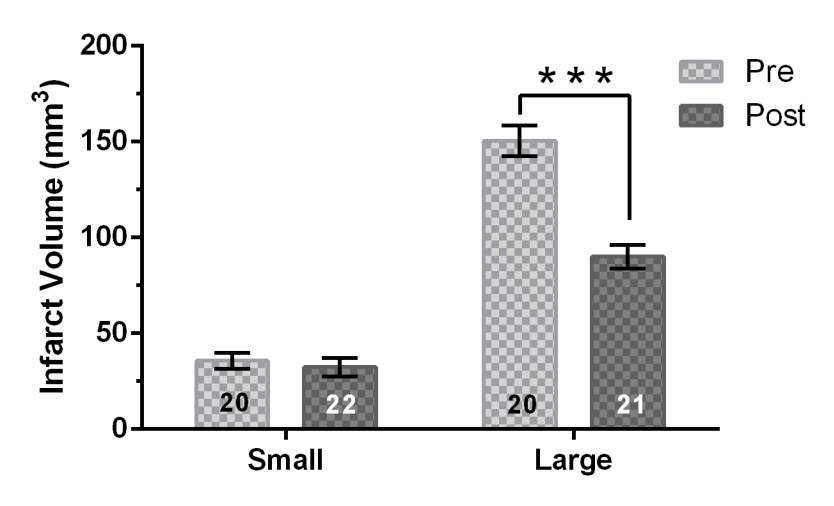

Figure 18 Supplemental. Infarct Volume of small and large strokes in pre-conditioning versus post-conditioning. Within the large strokes, rats in the pre-conditioning (light gray) group has significantly larger strokes than the post-conditioning (dark gray) group, independent of RIC. Pre-conditioning and post-conditioning was 18h before and 4h after stroke, respectively. Infarct volume was measured 24h post-stroke with MRI. Number of animals per group is indicated in the bars. Values are estimated marginal means ± SEM. P≤ .001(***).