ROLE OF MATRIX METALLOPROTEASE 3 AND TISSUE PLASMINOGEN ACTIVATOR IN MEDIATING NEUROVASCULAR INJURY AND WORSENING FUNCTIONAL OUTCOMES IN HYPERGLYCEMIC STROKE

By

SHERIF SHOKRY ABDELHALIM HAFEZ

(Under the Supervision of Adviye Ergul)

ABSTRACT

Ischemic stroke is a leading cause of death and disability. The incidence of hyperglycemia (HG) in acute ischemic stroke (AIS) patients exacerbates the cerebral hemorrhage and worsens the functional outcomes. It has been shown that patients with acute HG experience 5patients. Tissue plasminogen activator (tPA) is the only FDA approved thrombolytic therapy for AIS patients. However, its use in the clinical setting is limited due to its narrow therapeutic window and increased risk of intracerebral hemorrhage (ICH). Moreover, HG was shown to exacerbate the tPA induced hemorrhagic transformation (HT). The underlying mechanisms through which hyperglycemia exacerbates HT and worsens the clinical outcomes after stroke, especially with the administration of tPA are not fully understood. Matrix metalloproteinase 3 (MMP3) is an endopeptidase with broad substrate specificity that can target and degrade all the components of the neurovascular unit. It was previously shown that MMP3 is the critical mediator of the tPA induced ICH after stroke. However, the role of MMP3 in mediating HT in hyperglycemic stroke was not previously studied. Accordingly, we aimed in our work to investigate the impact of the interaction between HG and tPA on HT and stroke outcomes, elucidating the deleterious role of

MMP3. We showed that the degree of the neurovascular injury after stroke is dependent on the severity of HG and that the interaction between tPA and acute HG exacerbates the HT and worsens the functional outcomes independent of the method of reperfusion. Furthermore, we are the first to point out the role of MMP3 in exacerbating the vascular injury and HT in hyperglycemic stroke. We showed that the pharmacological inhibition and focal knockdown of MMP3 in the brain significantly reduced the HT and improved the outcomes. Our findings provide a better understanding of the mechanisms involved in mediating the HG induced HT after stroke and points out MMP3 as a novel and potential therapeutic target in hyperglycemic stroke and that its inhibition can reduce the HG induced HT and provide a potential clinical solution for safer administration of tPA.

INDEX WORDS: Ischemic Stroke; Hyperglycemia; Matrix Metalloprotease 3; Tissue Plasminogen Activator; Neurovascular Injury; Hemorrhagic Transformation.

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DEDICATION

I dedicate this thesis to my Family. My enthusiastic, caring and devoted mother who kept pushing me through all my life on the track of success and promotion. She has never kept an effort and she was always keen to make me the best ever. My beautiful sisters who gave me all the love and joy in the world. Though they are younger than me, they were always supportive and caring. And my father, the figure that imbued my personality with the manhood traits. He's always been a giving father who guides and gives the sincere advice and at the same time an elder friend whom I shared with unforgettable moments of joy.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Effect of Hyperglycemia on Stroke Outcomes- Clinical Evidence

Ischemic stroke is a leading cause of disability and becoming the fourth leading cause of death after heart, cancer and respiratory disease [1, 2]. As of 2015, stroke even dropped to the fifth leading cause of death after swapping positions with unintentional injuries according to a recent report by the American Heart Association (AHA). The incidence of cerebral ischemia triggers several multifactorial pathological cascades that cause vasogenic edema, disruption of the blood brain barrier, intracranial hemorrhage and neuronal death. Admission hyperglycemia (HG) is a frequent finding in patients admitted to the hospital with acute ischemic stroke, such that, almost 50% of stroke patients present to the hospital with HG. Admission HG was found to exacerbate the neurovascular damage, worsen functional outcome and increase the mortality rate. Around 40% of these patients develop acute HG as a stress response and it was shown that these patients have the least favorable functional outcome, longer in-hospital stay and higher risk of mortality when compared to normoglycemic or even diabetic stroke patients [3-6].

The high incidence of acute HG after the onset of stroke may be due to pre-existing abnormalities in glucose metabolism. First, HG is most probably developed after stroke as stress response that involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis [7, 8]. This leads to increased levels of circulating glucocorticoids (cortisol) and activation of the sympathetic autonomic nervous system, resulting in increased release of catecholamines. These increased levels of stress hormones lead to up-regulation of many

metabolic processes like glycogenolysis and gluconeogenesis which in turn increase glucose production. Moreover, the increased level of epinephrine inhibits the binding of insulin to its receptors. This inhibits the glucose transport into the cells and leads to the development of insulin resistance with hyperinsulinemia. Second, cerebral ischemia triggers a massive inflammatory response and leads to the release of a burst of inflammatory cytokines like tumor necrosis factor α (TNF α) and interleukin 1β (Il1β). These cytokines activate the HPA axis and promote insulin resistance and consequently increase blood glucose levels (Figure 1.1).

A large body of evidence from clinical trials shows the deleterious impact of HG on stroke outcomes. It has been shown in the TOAST trial that higher admission BG was associated with worse clinical outcomes in patients with non-lacunar stroke [6]. Admission HG (>8mmol/l) was shown to be an independent predictor of poor neurological outcomes and increased rate of symptomatic intracranial hemorrhage (SICH) in the NINDS and ATLANTIS trials [9, 10]. The ECASSII study showed that persisting HG for 24 hours or more is an independent predictor of poor clinical outcomes, ICH and death in AIS patients [11]. The only FDA approved therapeutic option for these patients is tissue plasminogen activator (tPA). However, its use in the clinical setting is limited due to its narrow therapeutic window and increased risk of ICH. Unfortunately, HG was shown also to aggravate the tPA induced cerebral hemorrhage in AIS patients [9, 12-16].

It appears from the provided evidence that HG is detrimental during AIS. Accordingly, it might be beneficial to lower the BG levels cautiously during the first few hours to days. However, due to the narrow evidence for efficacy from clinical trials, there are only limited guideline recommendations for BG goals during acute stroke. The American Heart

Association (AHA) current guidelines recommend maintaining BG levels in the range of 140-180 mg/dl for AIS patients for the first few days. The European stroke organization guidelines recommend lowering BG with insulin to below 180 mg/dl. In this aspect, several clinical trials were conducted to examine the safety, feasibility and efficacy of BG lowering using insulin during AIS (Table 1.1). In 2010, McCormick et al showed that the use of GKI (glucose potassium insulin) infusion within 24 h of stroke lowered blood glucose without affecting the cerebral infarct growth [17]. The Treatment of HG in Ischemic Stroke (THIS) trial was conducted to test the feasibility and safety of aggressive HG correction with IV insulin targeting BG levels <130 mg/dl. The results from this trial showed that the use of IV insulin protocol was safe and feasible without major adverse events [18]. Also the Glucose Regulation in Acute Stroke Patients (GRASP) trial showed the safety and feasibility of using IV insulin infusion for tight BG control targeting BG levels 70-110 mg/dl [19]. However, these trials showed only the safety and feasibility of the use of insulin in controlling BG during AIS, but didn't test the efficacy of theses protocols on improving the outcomes. The Stroke Hyperglycemia Insulin Network Effort (SHINE) trial is a current ongoing trial that aims to test the efficacy and safety of the use of intensive care insulin infusion targeting BG of 80 -130 mg/dl. *Consistent with these findings, we are testing clinically relevant BG levels in this project.*

Table 1.1. Management of HG in Acute Ischemic Stroke

Clinical Trial	GIST	THIS	GRASP	SHINE
Aims	Determine whether treatment with GKI infusion to maintain euglycemia after stroke would reduce the death at 90 days.	Test the feasibility and tolerability of aggressive HG correction with IV insulin compared to usual care standards in acute stroke.	Assess the Feasibility and Safety of two insulin infusion protocols in AIS patients.	Determine the safety and efficacy of standard vs. intensive glucose control with insulin in hyperglycemic acute ischemic stroke patients.
Arms	Patients presenting within 24 h of stroke onset and with admission HG were randomly assigned to receive GKI infusion vs saline (control). GKI target: 72-126 mg/dl.	1- Aggressive: I.V infusion target BG<130 mg/dl. 2- Usual care: SQ insulin QID, BG < 200 mg/dl.	1- Usual care (Discretion of treating physician) – target 70-300 mg/dL 2- Loose control - target 70 – 200 mg/dL – insulin infusion. 3-Tight control – target 70 – 110 mg/dL – insulin infusion.	1- Intensive care: IV insulin infusion with target glucose 80-130 mg/dL. 2- Standard care: Sliding scale SQ insulin will be given up to 4 times per day based on glucose concentration to keep BG < 180 mg/dL.
Conclusion	No significant reduction in mortality at 90 days. The trial was stopped due to slow enrollment. However, GKI significantly reduced blood glucose.	The intravenous insulin protocol corrected hyperglycemia during acute cerebral infarction significantly better than usual care without major adverse events. The Intensive insulin control was safe and feasible.	Insulin infusion for patients with acute ischemic stroke is feasible and safe.	Still ongoing trial and recruiting patients to assess safety and efficacy of tight BG control using insulin infusion.

Effect of Hyperglycemia on Stroke outcomes – Experimental Evidence

Many experimental studies showed the detrimental effects of HG on stroke outcomes in different animal models. From the pioneers in this field, de Courten-Mayers et al showed in 1988 that HG enlarges the infarct size after cerebrovascular occlusion in cats [20]. Other groups studied this also in rabbits and the most prominent candidates for these studies were rodents, whether rats or mice [21-26]. In 2007, Kamada et al showed that severe (400 mg/dl) but acute HG induced by STZ injection 3 days prior to stroke significantly increased the infarct size and blood brain barrier (BBB) disruption when compared to control rats [27]. In 2011, Cipolla et al showed that HG in rats subjected to 2h ischemia and 2h reperfusion increased BBB permeability and edema in the brain [28]. Moreover, it was shown recently by our group in 2011 that even the mild elevations in BG levels (140-200 mg/dl) significantly increased the vascular injury and HT in rats [29, 30]. In the same year, Xing et al showed that HG significantly increased the BBB permeability and HT without affecting the infarct size in rats and Won el al showed that HG exacerbated BBB disruption, HT and neuronal injury [24, 31]. Recently in 2015, it was shown by our group that the severity of HG is a determinant of the neurovascular injury after stroke. We showed that mild HG significantly exacerbated the HT and worsened the functional outcomes without further increasing the infarct size. However, severe HG not only exacerbated the vascular injury but also increased the neuronal death and infarct size [30]. Our findings demonstrate the sensitivity of the vasculature and its vulnerability to even subtle elevations in blood glucose levels. Therefore, as we proceed through the advances of stroke research, the role of the vasculature and HT in worsening stroke outcomes should be considered and the poor stroke outcomes should not be attributed only to the neuronal

injury. We have recently reviewed that large body of evidence from experimental studies showed that HG increases the infarct size, edema in the brain, vascular injury and HT, BBB disruption and worsens the functional outcomes after stroke [32]. However, *the underlying mechanisms contributing to this increased risk of vascular injury and HT in HG are not fully understood and this constitutes a significant deficit in hyperglycemic stroke research.*

Potential Mechanisms Contributing to the Neurovascular Injury in Hyperglycemic Stroke

HG aggravates the ischemic injury through several interconnected potential mechanisms (Figure 1.2).

1- HG impairs recanalization.

HG stimulates coagulation by increasing the production of thrombin and activating the tissue factor pathway. Together with hyperinsulinemia, HG reduces the fibrinolytic activity of tPA by increasing the production of plasminogen activator inhibitor1 PAI-1[33].

2- HG decreases reperfusion.

HG is associated with decreased reperfusion to the ischemic tissue, increased infarct volumes and worse outcomes [34-37]. Augmented production of reactive oxygen species (ROS), like superoxide anions and peroxynitrite, exacerbates the reperfusion injury in hyperglycemic stroke [36-38]. These deleterious effects of HG appear to depend on the presence of reperfusion [9, 13].

Back in 1997, Quast et al showed that hyperglycemic rats showed more reduction in cerebral blood flow (CBF) than normoglycemic ones. Even after recanalization, CBF of hyperglycemic rats was still less than their normoglycemic counterparts [36]. HG seems to cause reduction in CBF through inhibition of vasodilatation. HG was shown to increase the production of protein kinase C (PKC) which in turn reduces the expression of nitric oxide synthase and increases the activity of NADPH oxidase [39]. This reduces the production of nitric oxide and increases the production of superoxide, an effect that would collectively inhibit the nitric oxide dependent vasodilatation.

3- HG worsens the reperfusion injury.

Although restoration of blood flow is important for the salvage of the penumbra, reperfusion itself can induce injury and hyperglycemia can exacerbate this injury [36, 37, 40]. The mediators of the reperfusion injury are mainly oxidative stress and inflammatory response, and it seems that HG aggravates these two processes. HG activates protein kinase C (PKC) leading to activation of NADPH oxidase and increased superoxide production [38]. ROS can damage various cell components of the BBB including proteins, lipids and DNA, leading to BBB disruption, edema and increased hemorrhage [27].

- <u>Sources and generation of ROS after cerebral ischemia/reperfusion and HG [41]:</u>

1- Oxygen radicals are produced during the enzymatic conversion after stroke, such as the cyclooxygenase dependent conversion of arachidonic acid to prostanoids.

2- The formation of mitochondrial permeability transition pore leads to a burst of free oxygen radicals and release of pro-apoptotic molecules.

3- The inflammatory response triggered after ischemia leads to the generation of free radicals.
4- HG causes activation of protein kinase C (PKC) which leads to increased production of superoxide anion through activation of NADPH oxidase. HG also leads to increased glucose metabolism and activation of the hexoseamine pathway which leads to increased NADH production and increased electron transport through the electron transfer chain in the mitochondria. This leads to electron transfer to molecular oxygen converting it to superoxide anion.
5- Increased neuronal and endothelial nitric oxide synthases (nNOS and eNOS) in the early phase and inducible NOS (iNOS) in the late phase after ischemia leads to increased generation of nitric oxide (NO).
6- The high levels of both superoxide and nitric oxide lead to the formation of the deleterious peroxynitrite ($ONOO^{\cdot}$).

The high level of ROS species after stroke is highly detrimental. The produced superoxide is highly toxic and can damage the various components of the BBB, increase the neuronal death and consequently increase the ischemic lesion [42]. Moreover, ROS are believed to activate MMPs either directly (through oxidation, s-glutathilation or s-nitrosylation) or indirectly through activating a cascade of downstream signaling pathways that finally activates NFκB and leads to upregulation of MMPs [42-44]. In 2000, Morita-Fujimura et al showed that SOD1 deficient mice displayed increased expression of MMP9 and transgenic mice overexpressing SOD1 showed decreased lesion size and reduced MMP9 activity [45]. This explains the role of oxidative stress and ROS in mediating BBB disruption through activating MMPs.

- Role of peroxynitrite in hyperglycemic ischemic injury.

In 2001, Ste-Marie et al reported that nitrotyrosine immunoreactivity was more prominent under hyperglycemic conditions compared to normoglycemic ones after focal cerebral ischemia [46]. This was also associated with high levels of iNOS. So it appears that HG increases the production of superoxide and NO setting the stage ready for increased production of the noxious peroxynitrite.

Peroxynitrite is a potent oxidant and nitrating agent that can cause lipid peroxidation, protein nitration and DNA oxidation. Thus, it is highly involved in BBB impairment, edema formation, increased vascular injury and HT. All of these effects are aggravated by HG [38, 41, 47-49]. *In this proposal, we are addressing the role of peroxynitrite in activating MMP3 through tyrosine nitration as a potential mechanism underlying the HG mediated vascular injury and HT.*

- Role of inflammatory cytokines.

Cerebral ischemia/reperfusion triggers a massive inflammatory response and stimulates the release of inflammatory cytokines, mainly TNFα and IL_1B. This increases the production of adhesion molecules, neutrophil infiltration and adhesion to the cerebrovascular endothelium and stimulates further production of superoxide [41]. These inflammatory responses further increase BBB impairment and edema formation. HG exacerbates these inflammatory responses after stroke and leads to worse outcomes.

4- HG increases anaerobic glycolysis.

HG was shown to upregulate the process of anaerobic glycolysis leading to accumulation of lactic acid and creation of dysfunctional pH homeostasis, which in turn exacerbates the brain damage [50]. These findings were supported by the observation that HG correlates positively with increased cerebral lactate concentration and reduced penumbral salvage in human [5].

Furthermore, HG worsens the vascular injury through increasing the edema formation, vascular permeability and BBB disruption. These effects were attributed to the HG induced increase in the production of superoxide anion and MMP9 [27]. Past experimental studies related to hyperglycemic stroke induced ischemia reperfusion injury by suture occlusion model of stroke in severe clinically irrelevant hyperglycemic levels [27, 51, 52]. *To address this gap, in our studies we used a combination of different models of stroke (suture vs embolic stroke with or without tPA therapy) and clinically relevant blood glucose levels.*

Mediators of Hemorrhagic Transformation

HT may develop spontaneously or as a result of thrombolytic therapy by tPA in ischemic stroke. Better understanding of the mechanisms of HT is clinically very significant, not only for the primary prevention of HT but also for safer administration of tPA. The major mediators of HT in stroke are tPA and MMPs.

1. Tissue plasminogen activator (tPA).

tPA is the only FDA approved thrombolyic therapy for AIS patients for restoring the blood flow and salvaging the brain tissue. Although administration of tPA within 4.5 hours

or less of symptoms onset may improve the functional outcomes in patients, it still increases the risk of the incidence of symptomatic ICH by 10 folds. Furthermore, delayed reperfusion with tPA beyond 4.5 h is associated with increased risk of HT and brain injury with minimal benefit of re-establishing blood flow [53, 54]. Accordingly, there is a pressing need to discover new therapeutic strategies to reduce the tPA induced ICH and extend its therapeutic window without reducing its benefits.

TPA is a serine protease that is present predominantly in the blood. TPA is a double edge sword. In the blood it has desirable effects where it exerts a thrombolytic action to dissolve the formed blood clots and restore the blood flow to the ischemic brain tissue after stroke. However, it was shown that tPA can cross the damaged and even the intact BBB and reach the brain parenchyma where it exerts many undesirable neurotoxic effects [55, 56]. In the blood, tPA has a fibrinolytic activity where it functions to convert the inactive plasminogen into active plasmin. Its activity in the blood is regulated by plasminogen activator inhibitor-1 (PAI-1). In the brain, the activity of tPA is regulated by neuroserpin (a serine protease inhibitor in the brain) [57, 58].

- <u>Neurotoxicity of tPA.</u>

It is well documented in the literature that increased activity of endogenous tPA or administration of exogenous tPA after stroke increases the disruption of the BBB, neuronal death and hemorrhagic transformation. tPA mediates it neurotoxic effect through its action on the N-methyl D-aspartate (NMDA) receptors where it increases Ca^{2+} ion influx into the tissue, leading to excitotoxic effects and neuronal death [59, 60].

- tPA disrupts BBB.

TPA can cause degradation of the tight junction proteins and disruption of the BBB either directly through its proteolytic activity, or indirectly through activation of other proteases, mainly MMPs.

In 2003, Yepes et al showed that IV administration of exogenous tPA significantly increased the levels of MMP9 in the ischemic brain in rodents and this was associated with increased BBB permeability [61]. Tsuji et al showed in 2005 that $tPA^{-/-}$ (tPA deficient) mice showed a significant decrease in MMP9 levels in ischemic brains and exogenous administration of tPA in those animals restored MMP9 to WT levels [62]. This shows that tPA may be playing a role in MMP9 expression and activation. It was shown also that tPA can activate other MMPs, like MMP3, through which it can cause BBB disruption and exacerbates hemorrhage in the brain after stroke [63, 64].

Clinical data have shown that brain levels of MMP9 are elevated in AIS patients, and tPA treatment caused further increase in these levels [65-67]. Consequently, MMP9 level in the blood has emerged as a promising biomarker for human stroke, as levels of MMP9 correlate with poor neurologic outcomes and hemorrhagic complications.

- Clinical use of tPA.

TPA is the only FDA approved thrombolytic therapy for AIS patients but in spite of its beneficial effects in dissolving the blood clots and restoring the blood flow to the ischemic tissue, its use in the clinical setting is limited due to its narrow therapeutic window and increased risk of ICH. Many clinical trials were conducted to assess the efficacy and safety of tPA administration in AIS patients. In 2010, Lees et al conducted a pooled analysis study

to examine the effect of time to treatment with tPA after onset of stroke on therapeutic benefits and clinical outcomes. This study involved the findings of the NINDS1, NINDS 2, ATLANTIS A, ATLANTIS B, ECASS I, ECASS II, ECASS III and EPITHET trials. This study showed that the odds of clinical favorable outcomes at 3 months (defined as modified Rankin score of 0 or 1) increased as the onset of stroke to treatment time (OTT) decreased, with no clinical benefits after 270 minutes (4.5 hours). tPA administration increased the intracerebral hemorrhage (ICH) significantly when compared to the control group with no relation to OTT. The study showed also that there was an increase in mortality as OTT increases [54]. The findings from these clinical trials indicate that tPA is beneficial within 4.5 h after stroke onset and maximum efforts should be done to initiate the therapy as soon as possible of the symptoms onset. Beyond 4.5 h, the risk outweighs the benefit and no favorable outcomes are seen. This is mostly because of the death of the salvageable tissue (penumbra) and inability of the brain to recover.

- <u>tPA in hyperglycemic stroke</u>.

It was shown in the previously mentioned clinical studies (see above) that tPA increases the risk of ICH and the co-incidence of HG aggravates this effect [9, 12, 68]. In 2003, Alvarez-Sabin et al showed that HG is an independent predictor of poor clinical outcomes in AIS patients and Poppe et al in 2009 further supported this by showing that HG increases the rate of death, risk of symptomatic ICH and worse functional outcomes (Table 1.2) [12, 68]. In spite of the importance of tPA in the clinical setting, only few experimental studies were conducted to evaluate the impact of tPA on hyperglycemic stroke outcomes. Recently in 2011, Won et al [24] showed that acute HG exacerbated the brain damage in tPA treated

animals through increasing the infarct size, brain hemorrhage and BBB disruption. One year later, Fan et al showed that tPA administration in diabetic rats subjected to embolic stroke significantly increased the cerebral hemorrhage when compared to non-treated rats [21]. In 2013, the same group utilizing the same animal model showed that, the co-administration of minocycline with tPA after stroke significantly reduced brain infarction, edema and tPA induced HT [69]. The same group also showed that early glycemic control by insulin in combination with tPA reduced brain infarction, swelling and cerebral hemorrhage [70]. Recently it was published by our group (Hafez et al, 2015) that the combination of low dose tPA and acute HG significantly exacerbated the HT and worsened the functional outcomes in rats subjected to either suture or embolic occlusion [30]. All the above mentioned experimental studies corroborate the deleterious effect of HG on exacerbating the tPA induced HT (Table 1.3).

Table 1.2. Summary of Clinical Studies of HG and tPA

Clinical Studies		
Study	Purpose	Outcomes
Admission glucose level and clinical outcomes in the NINDS rt-PA Stroke Trial. Bruno et al, Neurology, 2002.	Analyze the relationship between admission glucose level and clinical outcomes from acute ischemic stroke.	High admission blood glucose levels were associated with undesirable clinical outcomes and significant increase in sICH.
Effects of admission hyperglycemia on stroke outcome in reperfused tissue plasminogen activator--treated patients. Alvarez-Sabin et al, Stroke, 2003.	Investigate the effect of admission HG on stroke outcomes in tPA treated patients.	Admission HG is an independent predictor of poor clinical outcomes despite of tPA induced recanalization.
Admission hyperglycemia predicts a worse outcome in stroke patients treated with intravenous thrombolysis. Poppe AY et al, Diabetes Care, 2009.	Investigate the effect of admission HG on long term stroke outcomes in tPA treated patients.	Admission HG was independently associated with increased risk of death, sICH and poor functional outcomes at 90 days.
Post-thrombolytic hyperglycemia and 3-month outcome in acute ischemic stroke. Putaala et al, Cerebrovasc Dis, 2011.	Investigate the impact of admission and persistent HG on Stroke outcomes after thrombolysis.	HG at admission and persisting for 48 h after tPA thrombolysis was associated with unfavorable clinical outcome, sICH and death.

Table 1.3. Summary of Experimental Studies of HG and tPA

Experimental Studies			
Study	Animal Model	Treatment	Outcomes
Hyperglycemia promotes tissue plasminogen activator-induced hemorrhage by Increasing superoxide production. Won et al, Ann Neurol, 2011.	SD rats, acute HG. Mechanical occlusion with micro-aneurysm clips for 90 minutes.	- tPA (10mg/kg IV) was infused 10 minutes before reperfusion.	tPA exacerbated brain infarct, edema and hemorrhage in hyperglycemic rats.
Tissue plasminogen activator treatment of stroke in type-1 diabetes rats. Ning et al, Neuroscience, 2012.	Type1 DM rats, embolic.	- tPA (10 mg/kg IV) at 2 h.	tPA significantly increased HT and brain swelling and failed to reduce the brain infarct.
A rat model of studying tissue-type plasminogen activator thrombolysis in ischemic stroke with diabetes. Fan et al, Stroke, 2012.	Type1 DM rats, embolic.	- tPA (10 mg/kg) at 1.5 h after stroke.	tPA slightly but significantly reduced the infarct size, but increased the cerebral hemorrhage.
Effects of minocycline plus tissue plasminogen activator combination therapy after focal embolic stroke in type 1 diabetic rats. Fan et al, Stroke, 2013.	Type1 DM rats, embolic.	- Minocycline (10 mg/kg IV) at 1 h. - tPA (10 mg/kg IV) at 1.5 h. - Minocycline (45 mg/kg IP) at 12 h	Combination of Minocycline with tPA significantly reduced brain infarction, brain swelling and tPA induced HT.
Early insulin glycemic control combined with tPA thrombolysis reduces acute brain tissue damages in a focal embolic stroke model of diabetic rats. Fan et al, Stroke, 2013.	Type1 DM rats, embolic.	- Insulin (2 U IV combined with 4 U SQ) at 1 h. - tPA (10 mg/kg IV) at 1.5 h.	Early use of insulin in combination with tPA significantly reduced brain infarction, brain swelling and tPA induced HT.
Comparative Analysis of Different Methods of Ischemia/Reperfusion in Hyperglycemic Stroke Outcomes: Interaction with tPA. Hafez et al, Transl Stroke Res, 2015.	1- Acute HG, Suture occlusion (90 min)/24h reperfusion. 2- Acute HG, Embolic model.	- tPA (1 mg/kg) at 2h after stroke.	The combination of tPA and HG exacacerbated the HT and worsened the functional outcomes more than each alone, independent of the method of reperfusion.

2. Matrix metalloproteases (MMPs).

MMPs encompass a family of zinc endopeptidases that can target all the components of the mammalian CNS. MMPs play both detrimental and beneficial roles in ischemic brains after stroke. During the early acute phase of stroke injury, MMPs have detrimental effects on the brain. They cause degradation of tight junction (claudin-5, occludin) and basal lamina (fibronectin, laminin, gelatin and proteoglycans) proteins. This leads to disruption in the BBB integrity, leakage, leukocyte infiltration, extravasation of fluids and plasma into brain parenchyma, edema and brain hemorrhage. In contrast, MMPs appear to play an important role in repairing the brain damage during the late phase of recovery through mediating the neurovascular unit remodeling [55, 71]. Accordingly and to play those two different roles, MMPs exhibit differential temporal and spatial expression within the neurovascular unit during the acute injury versus the delayed recovery phases.

In a rat model of transient middle cerebral artery occlusion (tMCAO), MMP9 was found to co-localize with brain microvascular ECs within the first 24 hours. While at days 7-14, the signal was shifted to the periphery of the cortical infarction and localize mainly with neurons and astrocytes. MMP3 was shown to co-localize with the neurons during the first 24 h hours after acute stroke [63]. However, a recent study by Yang et al showed that MMP3 was co-localized with the newly formed blood vessels and pericytes 3 weeks after stroke without being detected in the neurons [72]. This redistribution of MMP9 and MMP3 may reflect their multiphasic role after ischemic stroke as they might be playing a pathological role in the early phase mediating BBB disruption, neuronal cell death and

hemorrhage and beneficial role during the late phase of recovery through mediating neurovascular remodeling [73].

- Structure and activation of MMPs.

All the enzymes of the big family of the MMPs comprise common structure features. Actually, the basic structure of all the MMPs comprise three functional domains, a predomain, prodomain and catalytic domain. They differ from each other according to an extra peptide called the hemopexin domain attached to the basic domain through a hinge. The hemopexin domain differs between MMPs to determine the substrate specificity. Expression of MMPs in the adult brain is very low to undetectable, however, clinical and experimental studies showed that several MMPs are upregulated and activated after ischemic stroke. MMPs are secreted in latent inactive forms called zymogen. Their latency is preserved through the interaction between the zinc ion in the catalytic domain and the thiol (SH) group of the cysteine residue on the pro-domain. For MMPs activation, cleavage of the zinc-cysteine bridge must take place in a process called the "cysteine switch". This leads to exposure of the catalytic domain and activation of the enzyme (Figure 1.3). Cleavage of the zinc cysteine bridge may take place through several pathways, but the best known are: 1- The proteolytic cleavage through the action of serine protease like plasmin or tPA. Many studies demonstrated the ability of some MMPs to activate others, like the ability of MMP3 to activate MMP9. 2- Oxidative stress and elevation of reactive oxygen species and reactive nitrogen species (Figure 1.4) [43, 74].

- Role of MMPs in biphasic opening of the BBB.

BBB opening correlates with the redistribution of tight junction (TJ) and adherent junction (AJ) proteins from plasma membrane to cytoplasm as well as reorganization of actin cytoskeleton. In the literature, experimental studies showed that MMP2 is more involved in the initial opening of the BBB, while MMP9 contributes to the second (delayed) opening of the BBB. In 2007, Yang et al showed that in a rat model of tMCAO, initial opening of the BBB occurs as early as 3h after reperfusion and it was associated with increased activity of MMP2 and increased degradation of TJ proteins (claudin-5 and occludin). However, further degradation of TJ proteins at 24 h was due to increased MMP9 activity and this was associated with exacerbated HT [75].

Role of MMP3: A New Player in HT

- Name and History.

The name Stromelysin denotes a metalloproteinase derived from stromal cells and has the ability to hydrolyze the extracellular matrix (ECM). The enzymatic activity was first reported in 1974 by Woessner and his group when they showed that Cathepsin D was not able to digest proteoglycan at pH 7.5 and they attributed this action to another neutral protease isolated from the extracts of the human articular cartilage. In 1983, Galloway et al isolated and purified the enzyme from the bone marrow as a neutral metalloproteinase that can degrade proteoglycan and called it proteoglycanase. Later in 1985, Chin et al identified the enzyme as a 51 kDa metal dependent proteinase secreted by rabbit synovial fibroblasts and active at neutral pH. This group was the first to name the enzyme "Stromelysin". In 1996, Wilhelm and Woessner identified stromelysin1 as the acid

metalloproteinase in the human cartilage (Handbook of proteolytic enzymes, Neil D. Rawlings 2012).

- MMP3 activity.

MMP3 has broad substrate specificity and can cleave a large number of ECM and non-ECM proteins like; proteoglycan, fibronectin, gelatin, laminin, elastin and type IV collagen and it can be distinguished from other collagenases by its inability to degrade collagen type1. MMP3 can also activate other metalloproteinases (mainly MMP8, 9 and 13).

Interestingly, in 2000, Arza et al showed that pro-MMP3 can bind to tPA and plasminogen and enhance the activation of plasminogen by about 20 folds. Findings from the literature suggest that MMP3 plays an important physiological role in matrix remodeling during developmental processes. It is involved in involuting mammary glands, placental growth, endometrium cycling, embryogenesis and wound healing. It also plays a role in many pathological processes as well, either directly or through activating other matrixins. It is involved in osteoarthritis, deposition of atherosclerotic plaques, gastrointestinal ulcers (e.g., Crohn's disease, peptic ulcers) and mammary gland carcinoma. MMP3 serum levels are highly elevated in patients with rheumatoid arthritis and it is considered a predictor of joint destruction in these patients (Handbook of proteolytic enzymes, Neil D. Rawlings 2012).

- MMP3 in ischemic injury and HT.

MMP3 can degrade the TJs and the ECM proteins, a process through which it can disrupt the integrity of the BBB, leading to brain edema and HT. In 2004, Sole et al showed that the expression of MMP3 is highly upregulated in neurons of the ischemic brains of rats subjected to tMCAO. MMP3 activity was also increased in the homogenates of the damaged brains after stroke and this was indicated by cleavage of the cerebral matrix protein, agrin, an MMP3 substrate. These results indicate that the expression and activity of MMP3 can be upregulated in the brain after stroke and lead to ECM degradation and BBB disruption [76]. In 2006, Gurney et al showed that MMP3 mediates BBB opening in neuroinflamation. After intracerebral injection of lipopolysaccharide (LPS), MMP3 knock out (KO) mice showed a significant decrease in TJ proteins (claudin-5, occludin) degradation and reduced neutrophil infiltration when compared to WT mice [77]. In 2007, Suzuki et al showed that MMP3 plays an important role in mediating tPA induced intracerebral hemorrhage (ICH). In a mouse model of MCAO, MMP3 levels were significantly increased after stroke and mice treated with tPA showed a significant increase in ICH compared to non-treated controls. MMP3 but not MMP9 deficient mice showed a significant reduction in the tPA induced ICH, suggesting that MMP3 plays more important role than MMP9 in mediating the tPA induced ICH. In addition, administration of GM1006 (a broad spectrum MMPs inhibitor) after tPA treatment reduced the ICH in WT but not in MMP3 deficient mice. MMP3 was expressed in neurons after stroke and the administration of tPA selectively induced the expression of MMP3 in endothelial cells. This was further supported by MMP3 expression in vessel like structures after tPA treatment [63]. These findings were further supported in 2009 when the same group showed in another study that

tPA induced MMP3 expression in cultured murine brain ECs. This induction was prevented by inhibition of either low density lipoprotein related proteins (LRP) or nuclear factor κB ($NF_{\kappa}B$). These findings indicate that tPA might be mediating HT and promoting BBB damage through MMP3 induction in endothelial cells through LRP/NFκB mediated pathway [64].

Collectively, MMP3 can be a critical mediator of HT after stroke, especially with tPA administration. It can be activated either through the proteolytic action of tPA and plasmin or through ROS species generated after the ischemic insult. Consequently, it can cause degradation of the BBB either directly or through the activation of other MMPs, like MMP9 leading to HT (Figure 1.5). In spite of the importance of MMP3 in mediating the vascular injury and HT after stroke, there is not a single experimental study that evaluated the role of MMP3 in hyperglycemic stroke. *Accordingly, this study was designed to establish a mechanistic link between HG, MMP3 and HT.*

MMPs in Clinical Studies

MMP2 and MMP9 are the most extensively studied MMPs involved in mediating the ischemic injury. In particular, several studies showed that MMP9 (gelatinase B) activity is significantly elevated in human brain tissue and serum after stroke [78-81]. Castellanos et al showed that high plasma MMP9 levels is considered an independent predictor of HT after stroke [66]. In a study correlating MMP9 plasma levels to the severity of stroke, Demir et al reported that MMP9 levels increased during the acute phase of stroke and was associated with worse outcomes and brain infarction [82]. A review of the literature by Ramos-Fernandez et al in 2011 revealed that higher MMP9 plasma levels correlates to

larger infarct volume, severity of stroke and worse functional outcomes. They showed also that high levels of MMP9 is a predictor of HT after thrombolytic therapy with tPA after stroke [83]. Accordingly and due to the important role of MMP9 in mediating the ischemic injury, many experimental studies were conducted to examine the impact of inhibition of MMP9 on improving the stroke outcomes. Different groups used pharmacological MMP inhibitors like BB-94 [84] and GM6001 [63] and they showed improved outcomes. However, minocycline (which is originally a broad spectrum tetracycline antibiotic) is the only one successfully translated to clinical trials. In 2010, Fagan et al showed that administration of minocycline is safe and well tolerated up to 10 mg/kg alone or in combination with tPA [85]. One year later, Switzer et al investigated the effect of minocycline on plasma levels of MMP9 in acute ischemic stroke in the Minocycline to Improve Neurological Outcome in Stroke (MINOS) trial and a comparison group. They showed that administration of minocycline was associated with reduced MMP9 plasma levels in tPA treated patients. The group interpreted this as minocycline may reduce the tPA induced HT in AIS patients subjected to thrombolytic therapy and improve outcomes [81]. In a combined laser microdissection and protein array study, Cuadrado et al showed that MMP2, MMP3 and MMP9 were all upregulated in the infarct core tissue of AIS patients compared to healthy control areas [86].

Figure legends

Figure 1.1. Schematic diagram representing the mechanisms through which acute HG is developed after the incidence of stroke.

Acute HG may occur after stroke as a stress response which is associated with activation of the HPA. This leads to increased release of stress hormones like cortisol, catecholamines and glucagon, which in turn upregulate the metabolic processes that increase blood glucose, like glycogenolysis and gluconeogenesis. On the other side, the ischemic insult is associated with a massive increase in the release of inflammatory cytokines TNFα, IL-1β and IL-6, which together with the elevated levels of catecholamines may cause insulin resistance and HG.

Figure 1.1. Schematic diagram representing the mechanisms through which acute HG is developed after the incidence of stroke.

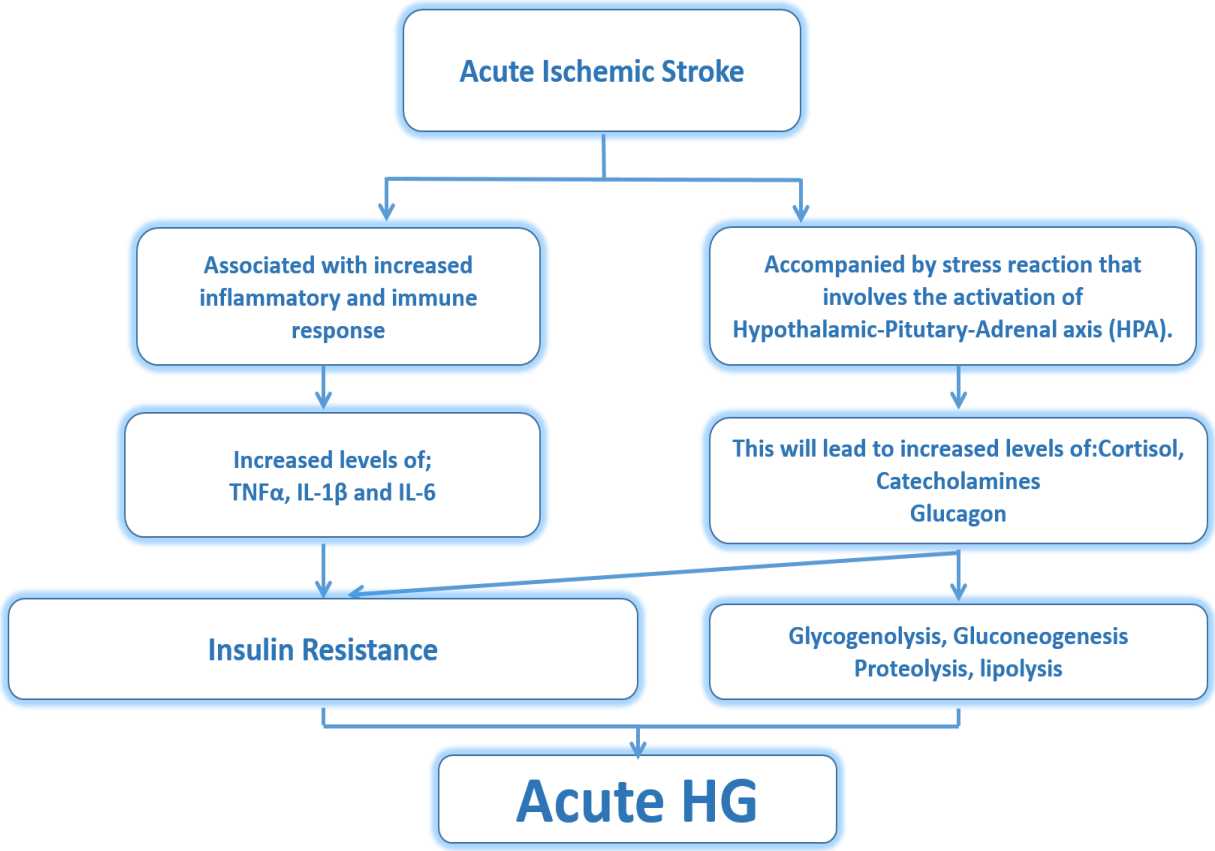


Figure 1.2. Schematic diagram representing the mechanisms through which HG exacerbates HT and worsens stroke outcomes.

eNOS: endothelial nitric oxide synthase; **NO:** nitric oxide; **VD:** vasodilatation; **NADPH:** Nicotinamide adenine dinucleotide phosphate; **PG:** prostaglandin; **TXA2:** thromboxane A2; **VC:** vasoconstriction; **ROS:** reactive oxygen species; **BBB:** blood brain barrier; **NFκB**: nuclear factor κB; **TNFα:** tumor necrosis factor α; **IL1B:** interleukin 1B; **HT:** hemorrhagic transformation.

Figure 1.2. Schematic diagram representing the mechanisms through which HG exacerbates HT and worsens stroke outcomes.

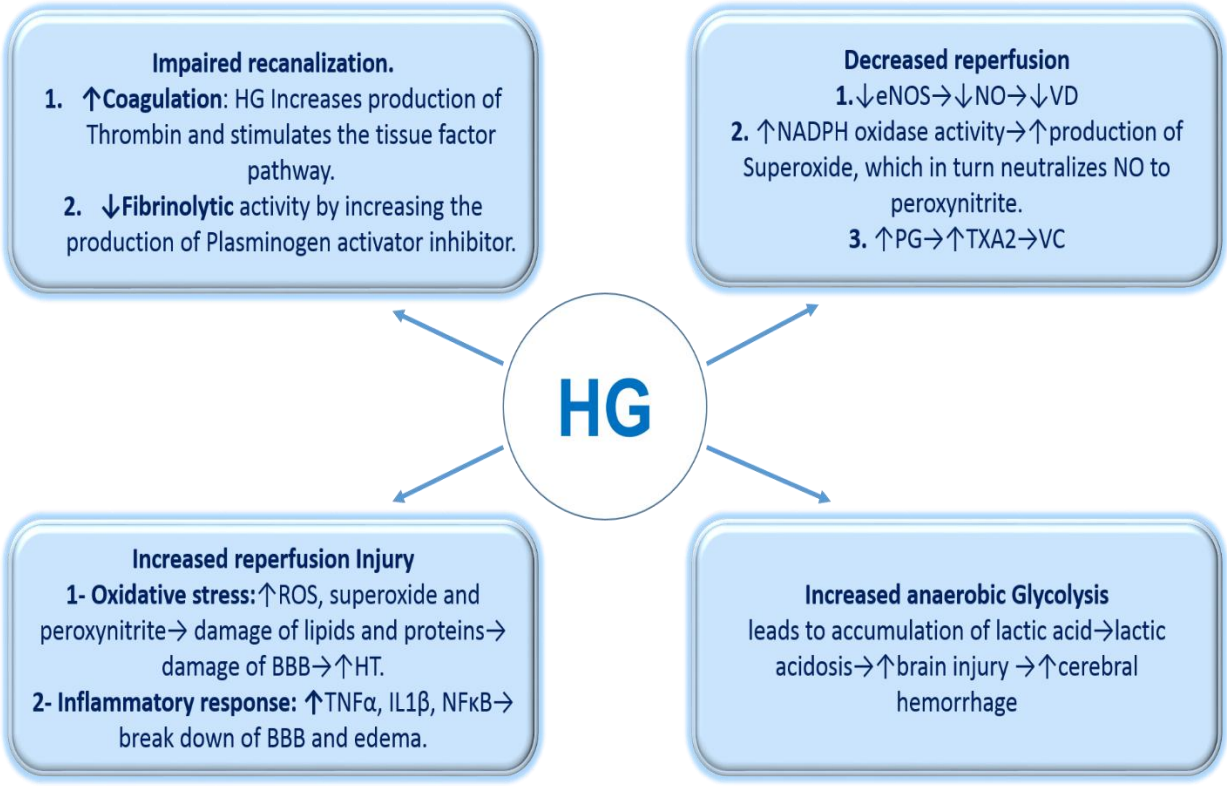


Figure 1.3. Schematic representation of the general structure of MMPs and the processes involved in their activation.

MMPs are basically composed of a pre-domain, a pro-domain with a cysteine residue and a catalytic domain with a zinc atom. MMPs are secreted in a latent inactive form. For activation, cleavage of the zinc-cysteine bridge must take place. The prodomain cleavage may take place through action of serine protease like plasmin or tPA causing direct proteolysis and activation of the enzyme. Reactive oxygen/nitrogen species may also cause disruption of the Zn-Cys bridge followed by autocatalysis leading to exposure of the catalytic domain and activation of the enzyme.

Figure 1.3. Schematic representation of the general structure of MMPs and the processes involved in their activation.

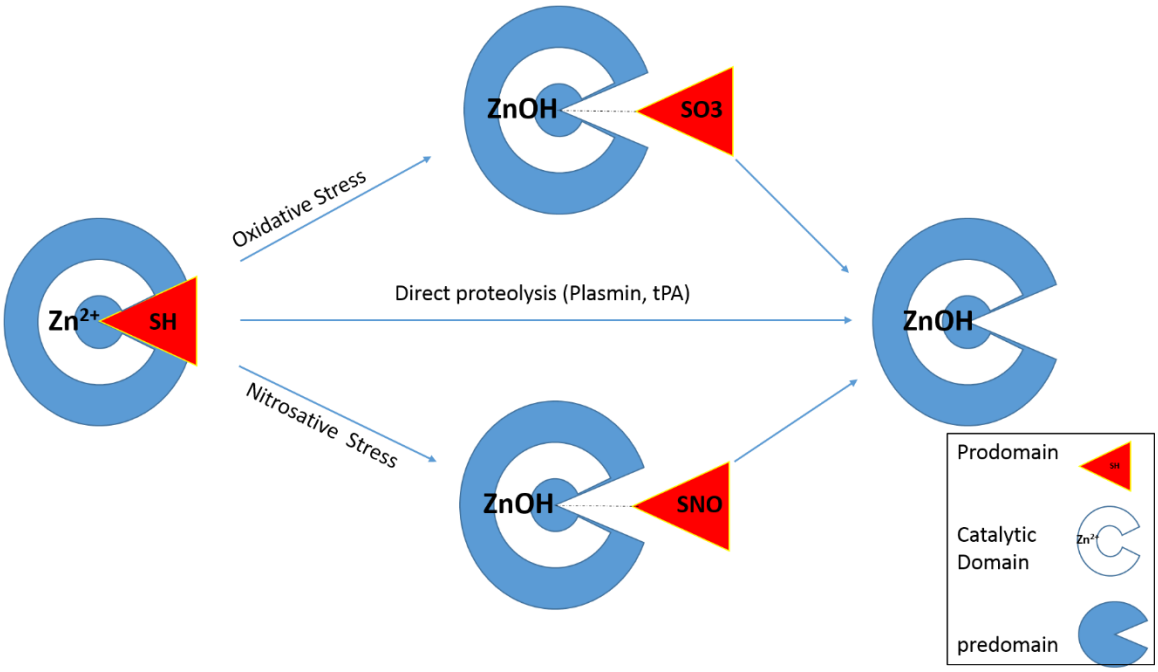


Figure 1.4. Schematic illustration of the different signaling pathways involved in MMPs activation.

The ischemic insult results in increased release of ROS and inflammatory cytokines that can convert the pro-MMP9 to the active MMP9. tPA activates MMP9 either through MMP3 mediated plasmin dependent pathway or through plasmin independent pathway through LRP (low density lipoprotein related protein) receptor and NFkB activation. tPA converts the inactive plasminogen to the active plasmin which in turn activate the pro-MMP3 to the active MMP3. The active plasmin activates the protein furin which activates the pro MT1-MMP to MT1-MMP (membrane type 1-MMP) which in turn activates MMP2. The active MMPs cause BBB disruption and HT.

Figure 1.4. Schematic illustration of the different signaling pathways involved in MMPs activation.

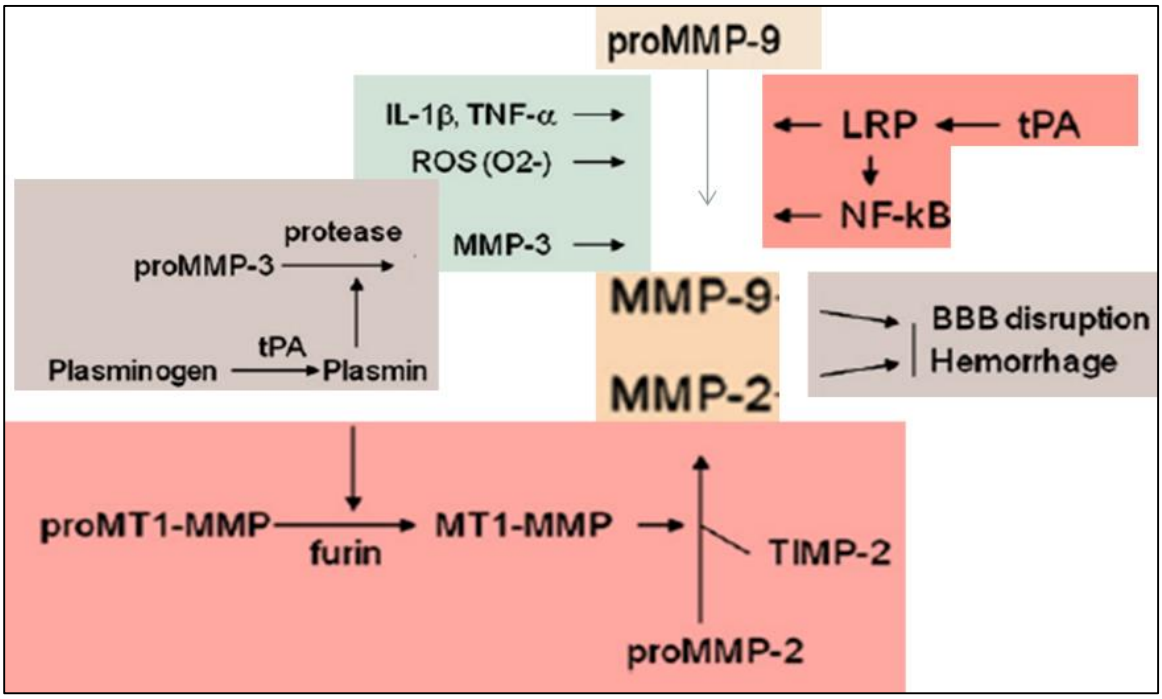
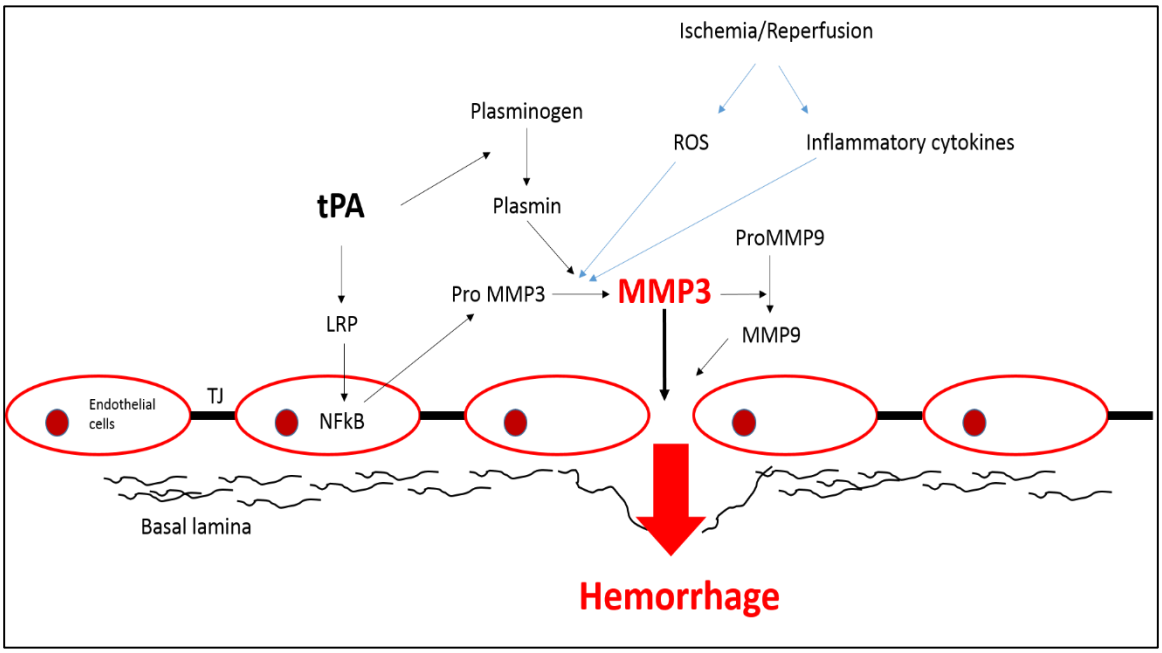


Figure 1.5. Schematic representation of the role of MMP3 in mediating HT after stroke.

tPA converts the inactive plasminogen to active plasmin which in turn converts the inactive pro-MMP3 to active MMP3. tPA also increases the production of MMP3 after stroke through the LRP/NFkB pathway. The ROS and inflammatory cytokines generated after the ischemic insult can also play a role in MMP3 activation. The active MMP3 can cause degradation of the basal lamina and TJ (tight junction) proteins leading to disruption of the BBB and HT, either directly through its proteolytic activity or through the activation of MMP9.

Figure 1.5. Schematic representation of the role of MMP3 in mediating HT after stroke.



PROBLEM STATEMENT AND SPECIFIC AIMS

Ischemic stroke is a leading cause of death and disability and it affects almost 800,000 individuals every year in the USA [1] . The incidence of HG was shown to worsen the stroke outcomes. The TOAST trial showed that higher admission blood glucose was associated with worse clinical outcomes in patients with non-lacunar stroke [6]. Admission HG was shown to be an independent predictor of poor neurological outcomes and increased rate of symptomatic intracranial hemorrhage (ICH) in the NINDS and ATLANTIS trials [9, 10]. The ECASS II trial also showed that HG persisting for 24 h or more is an independent predictor of poor clinical outcomes, ICH and death in AIS patients [11, 87]. Almost 50% of AIS patients are admitted to the hospital with elevated blood glucose levels and almost half of these patients develop acute HG without a previous history of diabetes. It was found that these patients suffer the least favorable clinical outcomes when compared to normoglycemic or even diabetic patients [3]. Moreover, HG increases the tPA induced cerebral hemorrhage after stroke. From these clinical findings, t is clear that HG worsens the vascular injury and increases bleeding after stroke, especially with the administration of tPA. *However, the underlying mechanism through which HG exacerbates the vascular injury and hemorrhagic transformation after stroke remains unclear and this constitutes a big gap in the field of hyperglycemic stroke.*

Findings from the experimental preclinical studies goes along with the clinical findings. It was shown that HG increases the infarct size, bleeding, edema and BBB disruption [21, 28, 29, 51, 88]. Clinical trials conducted to study the management of HG in ischemic stroke, like THIS (The Treatment of HG in Ischemic Stroke) [18] and GRASP (Glucose

Regulation in Stroke Patients) trials [19] demonstrated that blood glucose levels of AIS at admission was in the range of 160-240 mg/dl. However, previous preclinical studies evaluated the impact of HG on stroke outcomes in severely high and clinically irrelevant blood glucose levels (350-500 mg/dl) and most if not all of them employed the suture model to induce MCAO, which does not mimic what happens in patients. *This constitutes another gap in the field and to address this, we employed clinically relevant blood glucose levels in the hyperglycemia studies and we used different experimental stroke models (suture vs embolic, with and without human tPA dose) to closely mimic the clinical scenario.*

Hemorrhagic transformation may develop spontaneously or as a result of thrombolytic therapy with tPA after ischemic stroke and the coincidence of HG further exacerbates this injury. MMPs are considered the main mediators of HT after stroke. MMP2 and MMP9 are the most extensively studied and several groups showed that they are involved in BBB disruption, edema formation and HT [89-94]. In 2007, an interesting study showed that MMP3 plays a critical role in mediating the tPA induced intra-cerebral hemorrhage (ICH) and that MMP3, but not MMP9, deficient mice showed a reduction in tPA induced ICH. *However, in spite of its importance, the role of MMP3 in mediating the HT in hyperglycemic stroke was not previously studied and remained to be determined.*

HG is known also to increase the production of ROS after stroke. Peroxynitrite is one of the most potent oxidants and nitrating agents induced by HG and is known by its ability to cause lipid peroxidation and protein nitration. *Here, we are proposing the role of peroxynitrite in activating MMP3 through tyrosine nitration as a potential mechanism underlying the HG induced vascular injury and HT.*

Taken together, HG induces HT after stroke and injury is exacerbated with tPA administration. This creates a pressing need to develop new therapeutic approaches to extend the tPA administration window and reduce the HG and tPA induced HT after stroke. However, the limited understanding of the underlying mechanisms through which HG and tPA exacerbates HT after stroke creates a critical barrier against the development of new therapeutic targets and strategies. The overall objective of this project is to investigate the Impact of acute HG on stroke outcomes, especially with administration of tPA and identify MMP3 as a novel potential therapeutic target in hyperglycemic stroke through establishing a mechanistic link between HG, MMP3 and HT. Accordingly, we tested the major hypothesis that "Hyperglycemic reperfusion worsens stroke outcomes via MMP3 mediated disruption of vascular integrity".

SPECIFIC AIMS

<u>Aim 1:</u> Determine the role of MMP3 in exacerbated vascular injury in hyperglycemic ischemic stroke. In a clinically relevant animal model of acute HG, studies will test the hypotheses that:

1. Even mild elevation in blood glucose increases microvascular MMP3 activity and HT in hyperglycemic reperfusion injury.

2. HG-mediated MMP-3 activation and vascular injury is dependent upon the method of reperfusion. tPA amplifies reperfusion-mediated MMP3 activation in both suture or embolic occlusion models of stroke.

3. MMP3 inhibition prevents/reduces HT and improves functional outcome in hyperglycemic stroke.

Aim 2: Determine the mechanisms by which HG increases MMP3 activity and mediates loss of endothelial integrity. Using an in vitro oxygen/glucose deprivation (OGD) model, studies will test the hypotheses that:

1. Hyperglycemia and tPA synergistically increase MMP3 activation in brain microvascular endothelial cells after OGD.

2. Peroxynitrite-mediated nitration is the underlying mechanism of increased MMP3 activity in hyperglycemic reoxygenation.

BIBLIOGRAPHY

1. Roger, V.L., et al., *Heart disease and stroke statistics--2012 update: a report from the American Heart Association.* Circulation, 2012. **125**(1): p. e2-e220.
2. Roger, V.L., et al., *Heart disease and stroke statistics--2011 update: a report from the American Heart Association.* Circulation, 2011. **123**(4): p. e18-e209.
3. Capes, S.E., et al., *Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview.* Stroke, 2001. **32**(10): p. 2426-32.
4. Dungan, K.M., S.S. Braithwaite, and J.C. Preiser, *Stress hyperglycaemia.* Lancet, 2009. **373**(9677): p. 1798-807.
5. Parsons, M.W., et al., *Acute hyperglycemia adversely affects stroke outcome: a magnetic resonance imaging and spectroscopy study.* Ann Neurol, 2002. **52**(1): p. 20-8.
6. Bruno, A., et al., *Acute blood glucose level and outcome from ischemic stroke. Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators.* Neurology, 1999. **52**(2): p. 280-4.
7. Van den Berghe, G., *Novel insights into the neuroendocrinology of critical illness.* Eur J Endocrinol, 2000. **143**(1): p. 1-13.
8. Kruyt, N.D., et al., *Hyperglycemia in acute ischemic stroke: pathophysiology and clinical management.* Nat Rev Neurol, 2010. **6**(3): p. 145-55.
9. Bruno, A., et al., *Admission glucose level and clinical outcomes in the NINDS rt-PA Stroke Trial.* Neurology, 2002. **59**(5): p. 669-74.
10. Clark, W.M., et al., *The rtPA (alteplase) 0- to 6-hour acute stroke trial, part A (A0276g) : results of a double-blind, placebo-controlled, multicenter study. Thromblytic therapy in acute ischemic stroke study investigators.* Stroke, 2000. **31**(4): p. 811-6.
11. Yong, M. and M. Kaste, *Dynamic of hyperglycemia as a predictor of stroke outcome in the ECASS-II trial.* Stroke, 2008. **39**(10): p. 2749-55.
12. Alvarez-Sabin, J., et al., *Effects of admission hyperglycemia on stroke outcome in reperfused tissue plasminogen activator--treated patients.* Stroke, 2003. **34**(5): p. 1235-41.
13. Tanne, D., et al., *Markers of increased risk of intracerebral hemorrhage after intravenous recombinant tissue plasminogen activator therapy for acute ischemic stroke in clinical practice: the Multicenter rt-PA Stroke Survey.* Circulation, 2002. **105**(14): p. 1679-85.
14. Demchuk, A.M., et al., *Serum glucose level and diabetes predict tissue plasminogen activator-related intracerebral hemorrhage in acute ischemic stroke.* Stroke, 1999. **30**(1): p. 34-9.
15. Demchuk, A.M., et al., *Predictors of good outcome after intravenous tPA for acute ischemic stroke.* Neurology, 2001. **57**(3): p. 474-80.
16. Kase, C.S., et al., *Cerebral hemorrhage after intra-arterial thrombolysis for ischemic stroke: the PROACT II trial.* Neurology, 2001. **57**(9): p. 1603-10.
17. McCormick, M., et al., *Randomized, controlled trial of insulin for acute poststroke hyperglycemia.* Ann Neurol, 2010. **67**(5): p. 570-8.

18. Bruno, A., et al., *Treatment of hyperglycemia in ischemic stroke (THIS): a randomized pilot trial.* Stroke, 2008. **39**(2): p. 384-9.
19. Johnston, K.C., et al., *Glucose Regulation in Acute Stroke Patients (GRASP) trial: a randomized pilot trial.* Stroke, 2009. **40**(12): p. 3804-9.
20. Ford, L., et al., *Cerebral hemiatrophy--correlation of human with animal experimental data.* Pediatr Neurosci, 1988. **14**(3): p. 114-9.
21. Fan, X., et al., *A rat model of studying tissue-type plasminogen activator thrombolysis in ischemic stroke with diabetes.* Stroke, 2012. **43**(2): p. 567-70.
22. Kumari, R., et al., *The PPAR-gamma agonist, darglitazone, restores acute inflammatory responses to cerebral hypoxia-ischemia in the diabetic ob/ob mouse.* J Cereb Blood Flow Metab, 2010. **30**(2): p. 352-60.
23. Siemkowicz, E., A.J. Hansen, and A. Gjedde, *Hyperglycemic ischemia of rat brain: the effect of post-ischemic insulin on metabolic rate.* Brain Res, 1982. **243**(2): p. 386-90.
24. Won, S.J., et al., *Hyperglycemia promotes tissue plasminogen activator-induced hemorrhage by Increasing superoxide production.* Ann Neurol, 2011. **70**(4): p. 583-90.
25. Yan, T., et al., *Niaspan increases axonal remodeling after stroke in type 1 diabetes rats.* Neurobiol Dis, 2012. **46**(1): p. 157-64.
26. Ye, X., et al., *Niaspan enhances vascular remodeling after stroke in type 1 diabetic rats.* Exp Neurol, 2011. **232**(2): p. 299-308.
27. Kamada, H., et al., *Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction.* Stroke, 2007. **38**(3): p. 1044-9.
28. Cipolla, M.J., Q. Huang, and J.G. Sweet, *Inhibition of protein kinase Cbeta reverses increased blood-brain barrier permeability during hyperglycemic stroke and prevents edema formation in vivo.* Stroke, 2011. **42**(11): p. 3252-7.
29. Elgebaly, M.M., et al., *Neurovascular injury in acute hyperglycemia and diabetes: A comparative analysis in experimental stroke.* Transl Stroke Res, 2011. **2**(3): p. 391-398.
30. Hafez, S., et al., *Comparative Analysis of Different Methods of Ischemia/Reperfusion in Hyperglycemic Stroke Outcomes: Interaction with tPA.* Transl Stroke Res, 2015.
31. Xing, Y., et al., *Effects of deferoxamine on brain injury after transient focal cerebral ischemia in rats with hyperglycemia.* Brain Res, 2009. **1291**: p. 113-21.
32. Hafez, S., et al., *Hyperglycemia, Acute Ischemic Stroke, and Thrombolytic Therapy.* Transl Stroke Res, 2014.
33. Pandolfi, A., et al., *Acute hyperglycemia and acute hyperinsulinemia decrease plasma fibrinolytic activity and increase plasminogen activator inhibitor type 1 in the rat.* Acta Diabetol, 2001. **38**(2): p. 71-6.
34. Duckrow, R.B., D.C. Beard, and R.W. Brennan, *Regional cerebral blood flow decreases during chronic and acute hyperglycemia.* Stroke, 1987. **18**(1): p. 52-8.
35. Kawai, N., et al., *Hyperglycemia induces progressive changes in the cerebral microvasculature and blood-brain barrier transport during focal cerebral ischemia.* Acta Neurochir Suppl, 1998. **71**: p. 219-21.

36. Quast, M.J., et al., *Perfusion deficit parallels exacerbation of cerebral ischemia/reperfusion injury in hyperglycemic rats.* J Cereb Blood Flow Metab, 1997. **17**(5): p. 553-9.
37. Yip, P.K., et al., *Effect of plasma glucose on infarct size in focal cerebral ischemia-reperfusion.* Neurology, 1991. **41**(6): p. 899-905.
38. Suh, S.W., et al., *Glucose and NADPH oxidase drive neuronal superoxide formation in stroke.* Ann Neurol, 2008. **64**(6): p. 654-63.
39. Inoguchi, T., et al., *High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells.* Diabetes, 2000. **49**(11): p. 1939-45.
40. Wei, J., N.C. Huang, and M.J. Quast, *Hydroxyl radical formation in hyperglycemic rats during middle cerebral artery occlusion/reperfusion.* Free Radic Biol Med, 1997. **23**(7): p. 986-95.
41. Bemeur, C., L. Ste-Marie, and J. Montgomery, *Increased oxidative stress during hyperglycemic cerebral ischemia.* Neurochem Int, 2007. **50**(7-8): p. 890-904.
42. Lehner, C., et al., *Oxidative stress and blood-brain barrier dysfunction under particular consideration of matrix metalloproteinases.* Antioxid Redox Signal, 2011. **15**(5): p. 1305-23.
43. Florczak-Rzepka, M., et al., *Matrix metalloproteinases in human spontaneous intracerebral hemorrhage: an update.* Cerebrovasc Dis, 2012. **34**(4): p. 249-62.
44. Lakhan, S.E., et al., *Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke.* Front Neurol, 2013. **4**: p. 32.
45. Morita-Fujimura, Y., et al., *Overexpression of copper and zinc superoxide dismutase in transgenic mice prevents the induction and activation of matrix metalloproteinases after cold injury-induced brain trauma.* J Cereb Blood Flow Metab, 2000. **20**(1): p. 130-8.
46. Ste-Marie, L., et al., *Immunohistochemical detection of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase following hyperglycemic focal cerebral ischemia.* Brain Res, 2001. **918**(1-2): p. 10-9.
47. Bemeur, C., et al., *Dehydroascorbic acid normalizes several markers of oxidative stress and inflammation in acute hyperglycemic focal cerebral ischemia in the rat.* Neurochem Int, 2005. **46**(5): p. 399-407.
48. Li, S., J. Zheng, and S.T. Carmichael, *Increased oxidative protein and DNA damage but decreased stress response in the aged brain following experimental stroke.* Neurobiol Dis, 2005. **18**(3): p. 432-40.
49. Muralikrishna Adibhatla, R. and J.F. Hatcher, *Phospholipase A2, reactive oxygen species, and lipid peroxidation in cerebral ischemia.* Free Radic Biol Med, 2006. **40**(3): p. 376-87.
50. Schurr, A., *Lactate: the ultimate cerebral oxidative energy substrate?* J Cereb Blood Flow Metab, 2006. **26**(1): p. 142-52.
51. de Courten-Myers, G.M., et al., *Hemorrhagic infarct conversion in experimental stroke.* Ann Emerg Med, 1992. **21**(2): p. 120-6.
52. Martini, S.R. and T.A. Kent, *Hyperglycemia in acute ischemic stroke: a vascular perspective.* J Cereb Blood Flow Metab, 2007. **27**(3): p. 435-51.

53. Fugate, J.E. and A.A. Rabinstein, *Update on intravenous recombinant tissue plasminogen activator for acute ischemic stroke.* Mayo Clin Proc, 2014. **89**(7): p. 960-72.
54. Lees, K.R., et al., *Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials.* Lancet, 2010. **375**(9727): p. 1695-703.
55. Adibhatla, R.M. and J.F. Hatcher, *Tissue plasminogen activator (tPA) and matrix metalloproteinases in the pathogenesis of stroke: therapeutic strategies.* CNS Neurol Disord Drug Targets, 2008. **7**(3): p. 243-53.
56. Yepes, M., et al., *Tissue-type plasminogen activator in the ischemic brain: more than a thrombolytic.* Trends Neurosci, 2009. **32**(1): p. 48-55.
57. Gravanis, I. and S.E. Tsirka, *Tissue-type plasminogen activator as a therapeutic target in stroke.* Expert Opin Ther Targets, 2008. **12**(2): p. 159-70.
58. Miranda, E. and D.A. Lomas, *Neuroserpin: a serpin to think about.* Cell Mol Life Sci, 2006. **63**(6): p. 709-22.
59. Nicole, O., et al., *The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling.* Nat Med, 2001. **7**(1): p. 59-64.
60. Lopez-Atalaya, J.P., et al., *Toward safer thrombolytic agents in stroke: molecular requirements for NMDA receptor-mediated neurotoxicity.* J Cereb Blood Flow Metab, 2008. **28**(6): p. 1212-21.
61. Yepes, M., et al., *Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein.* J Clin Invest, 2003. **112**(10): p. 1533-40.
62. Tsuji, K., et al., *Tissue plasminogen activator promotes matrix metalloproteinase-9 upregulation after focal cerebral ischemia.* Stroke, 2005. **36**(9): p. 1954-9.
63. Suzuki, Y., et al., *Stromelysin-1 (MMP-3) is critical for intracranial bleeding after t-PA treatment of stroke in mice.* J Thromb Haemost, 2007. **5**(8): p. 1732-9.
64. Suzuki, Y., et al., *Tissue-type plasminogen activator (t-PA) induces stromelysin-1 (MMP-3) in endothelial cells through activation of lipoprotein receptor-related protein.* Blood, 2009. **114**(15): p. 3352-8.
65. Barr, T.L., et al., *Blood-brain barrier disruption in humans is independently associated with increased matrix metalloproteinase-9.* Stroke, 2010. **41**(3): p. e123-8.
66. Castellanos, M., et al., *Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke.* Stroke, 2003. **34**(1): p. 40-6.
67. Castellanos, M., et al., *Serum cellular fibronectin and matrix metalloproteinase-9 as screening biomarkers for the prediction of parenchymal hematoma after thrombolytic therapy in acute ischemic stroke: a multicenter confirmatory study.* Stroke, 2007. **38**(6): p. 1855-9.
68. Poppe, A.Y., et al., *Admission hyperglycemia predicts a worse outcome in stroke patients treated with intravenous thrombolysis.* Diabetes Care, 2009. **32**(4): p. 617-22.
69. Fan, X., E.H. Lo, and X. Wang, *Effects of minocycline plus tissue plasminogen activator combination therapy after focal embolic stroke in type 1 diabetic rats.* Stroke, 2013. **44**(3): p. 745-52.

70. Fan, X., et al., *Early insulin glycemic control combined with tPA thrombolysis reduces acute brain tissue damages in a focal embolic stroke model of diabetic rats.* Stroke, 2013. **44**(1): p. 255-9.
71. Cunningham, L.A., M. Wetzel, and G.A. Rosenberg, *Multiple roles for MMPs and TIMPs in cerebral ischemia.* Glia, 2005. **50**(4): p. 329-39.
72. Yang, Y., et al., *Early inhibition of MMP activity in ischemic rat brain promotes expression of tight junction proteins and angiogenesis during recovery.* J Cereb Blood Flow Metab, 2013. **33**(7): p. 1104-14.
73. Zhao, B.Q., et al., *Role of matrix metalloproteinases in delayed cortical responses after stroke.* Nat Med, 2006. **12**(4): p. 441-5.
74. Jin, R., G. Yang, and G. Li, *Inflammatory mechanisms in ischemic stroke: role of inflammatory cells.* J Leukoc Biol, 2010. **87**(5): p. 779-89.
75. Yang, Y., et al., *Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat.* J Cereb Blood Flow Metab, 2007. **27**(4): p. 697-709.
76. Sole, S., et al., *Activation of matrix metalloproteinase-3 and agrin cleavage in cerebral ischemia/reperfusion.* J Neuropathol Exp Neurol, 2004. **63**(4): p. 338-49.
77. Gurney, K.J., E.Y. Estrada, and G.A. Rosenberg, *Blood-brain barrier disruption by stromelysin-1 facilitates neutrophil infiltration in neuroinflammation.* Neurobiol Dis, 2006. **23**(1): p. 87-96.
78. Clark, W.M., et al., *Recombinant tissue-type plasminogen activator (Alteplase) for ischemic stroke 3 to 5 hours after symptom onset. The ATLANTIS Study: a randomized controlled trial. Alteplase Thrombolysis for Acute Noninterventional Therapy in Ischemic Stroke.* JAMA, 1999. **282**(21): p. 2019-26.
79. Montaner, J., et al., *Matrix metalloproteinase expression is related to hemorrhagic transformation after cardioembolic stroke.* Stroke, 2001. **32**(12): p. 2762-7.
80. Horstmann, S., et al., *Profiles of matrix metalloproteinases, their inhibitors, and laminin in stroke patients: influence of different therapies.* Stroke, 2003. **34**(9): p. 2165-70.
81. Switzer, J.A., et al., *Matrix metalloproteinase-9 in an exploratory trial of intravenous minocycline for acute ischemic stroke.* Stroke, 2011. **42**(9): p. 2633-5.
82. Demir, R., et al., *Relationship between plasma metalloproteinase-9 levels and volume and severity of infarct in patients with acute ischemic stroke.* Acta Neurol Belg, 2012. **112**(4): p. 351-6.
83. Ramos-Fernandez, M., M.F. Bellolio, and L.G. Stead, *Matrix metalloproteinase-9 as a marker for acute ischemic stroke: a systematic review.* J Stroke Cerebrovasc Dis, 2011. **20**(1): p. 47-54.
84. Pfefferkorn, T. and G.A. Rosenberg, *Closure of the blood-brain barrier by matrix metalloproteinase inhibition reduces rtPA-mediated mortality in cerebral ischemia with delayed reperfusion.* Stroke, 2003. **34**(8): p. 2025-30.
85. Fagan, S.C., et al., *Minocycline to improve neurologic outcome in stroke (MINOS): a dose-finding study.* Stroke, 2010. **41**(10): p. 2283-7.
86. Cuadrado, E., et al., *Vascular MMP-9/TIMP-2 and neuronal MMP-10 up-regulation in human brain after stroke: a combined laser microdissection and protein array study.* J Proteome Res, 2009. **8**(6): p. 3191-7.

87. Piironen, K., et al., *Glucose and acute stroke: evidence for an interlude.* Stroke, 2012. **43**(3): p. 898-902.
88. Elgebaly, M.M., et al., *Vascular protection in diabetic stroke: role of matrix metalloprotease-dependent vascular remodeling.* J Cereb Blood Flow Metab, 2010. **30**(12): p. 1928-38.
89. Asahi, M., et al., *Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94.* J Cereb Blood Flow Metab, 2000. **20**(12): p. 1681-9.
90. Asahi, M., et al., *Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia.* J Neurosci, 2001. **21**(19): p. 7724-32.
91. Atkinson, J.J., et al., *The role of matrix metalloproteinase-9 in cigarette smoke-induced emphysema.* Am J Respir Crit Care Med, 2011. **183**(7): p. 876-84.
92. Romanic, A.M., et al., *Matrix metalloproteinase expression increases after cerebral focal ischemia in rats: inhibition of matrix metalloproteinase-9 reduces infarct size.* Stroke, 1998. **29**(5): p. 1020-30.
93. Rosenberg, G.A., E.Y. Estrada, and J.E. Dencoff, *Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain.* Stroke, 1998. **29**(10): p. 2189-95.
94. Sumii, T. and E.H. Lo, *Involvement of matrix metalloproteinase in thrombolysis-associated hemorrhagic transformation after embolic focal ischemia in rats.* Stroke, 2002. **33**(3): p. 831-6.

CHAPTER 2

COMPARATIVE ANALYSIS OF DIFFERENT METHODS OF ISCHEMIA/REPERFUSION IN HYPERGLYCEMIC STROKE OUTCOMES: INTERACTION WITH TPA[1]

[1] Sherif Hafez, Md Nasrul Hoda, Xinyue Guo, Maribeth H. Johnson, Susan C. Fagan and Adviye Ergul. Transl Stroke Res. 2015 Jun; 6(3):171-80.

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COMPARATIVE ANALYSIS OF DIFFERENT METHODS OF ISCHEMIA/REPERFUSION IN HYPERGLYCEMIC STROKE OUTCOMES: INTERACTION WITH TPA

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Abstract

Acute hyperglycemia (HG) exacerbates reperfusion injury and aggravates tPA-induced hemorrhagic transformation (HT). Previous experimental hyperglycemic stroke studies employed very high blood glucose levels and exclusively used suture occlusion model to induce ischemia. Only few studies evaluated HG in embolic stroke and mostly involving the use of 10-fold higher dose of tPA than that is used in patients. However, the interaction between acute HG and low (human) dose tPA in different experimental models of stroke has never been reported. We first tested the impact of the severity of acute HG on stroke outcome. Building upon our findings, we then compared the impact of mild acute HG on neurovascular injury in rats subjected to suture or thromboembolic occlusion with and without low dose tPA. We assessed cerebral blood flow, neurobehavioral outcomes, infarction, hemorrhage and edema. tPA did not change the infarct size in either control or hyperglycemic animals when compared to no tPA groups. HG increased HT and worsened functional outcomes in both suture and embolic occlusion models. The combination of HG and tPA exacerbated the vascular injury and worsened the neurological deficits more than each individual treatment in both models. Our findings show that the interaction between HG and even low dose tPA has detrimental effects on the cerebrovasculature and functional outcomes independent of the method of reperfusion.

Introduction

Almost 40% of acute ischemic stroke (AIS) patients present with admission hyperglycemia (HG), an independent predictor of poor outcomes in AIS [1, 2]. Restoration of cerebral blood flow (CBF) to the ischemic brain is paramount for the salvation of the ischemic penumbra. However, reperfusion itself can induce brain injury through increased oxidative stress and inflammation. Moreover, HG exacerbates the reperfusion injury [3-6]. As recently reviewed, experimental models using various methods to induce hyperglycemia in different species recapitulated the clinical findings and showed that HG increases infarct size, brain swelling, hemorrhagic transformation (HT), blood brain barrier (BBB) disruption and neurological deficits [7-12]. However, most of these preclinical studies employed very high blood glucose levels and used a mix of diabetes and acute HG models. Given that patients that have HG with no prior history of diabetes suffer the least favorable clinical outcomes, in a recent study we compared the neurovascular injury after ischemic stroke in acute hyperglycemia and diabetes models. We reported that even a mild and acute elevation in blood glucose increases vascular injury and worsens functional outcome [9]. Since these past studies, including ours, almost exclusively used a suture occlusion model to induce stroke, which promptly restores CBF to the ischemic area, a direct comparison of the impact of the method of reperfusion on acute hyperglycemic stroke injury and outcome remained to be determined.

Tissue plasminogen activator (tPA) is the only FDA-approved therapy for AIS patients. Yet, its use in the clinical setting is limited due to its narrow therapeutic window and increased risk of cerebral bleeding, which is more profound under hyperglycemic conditions [13-15]. In spite of the importance of tPA in the clinical setting, only a handful

of studies involved tPA in experimental hyperglycemic models. Moreover, due to the differences in human and rodent fibrinolytic system, current embolic stroke models use 10 times greater dose of tPA than that is used in patients [7, 16-20]. Thus, in this translational study using suture and thromboembolic occlusion of middle cerebral artery (MCA), with and without human dose tPA in the hyperglycemic setting, we tested the hypothesis that even acute mild HG worsens the neurovascular injury and stroke outcomes irrespective of the method of reperfusion and that the use of tPA amplifies this injury.

Materials and Methods

Animal Models

The animals were housed at the Georgia Regents University animal care facility, which is approved by the American Association for Accreditation of Laboratory Animal Care. This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research and all protocols were approved by the institutional animal care and use committee.

Study 1. To determine the effect of the severity of hyperglycemia, male Wistar rats (Harlan Laboratories Inc., Indianapolis, IN) were assigned to 3 different groups: (1) Control/Normoglycemic (NG) (90 – 120 mg/dl), (2) Mild HG (BG 140 – 200 mg/dl), and (3) Severe HG (BG 240 – 350 mg/dl). Animals from all groups were subjected to 90

minutes MCA occlusion (MCAO) by the suture occlusion method and up to 24 hours reperfusion. 30% and 60% glucose solutions were used to achieve blood glucose levels of 140 - 200 mg/dl and 240 – 350 mg/dl, respectively. Acute HG was achieved by 2ml intraperitoneal (IP) injection of glucose solution 15 min before MCAO. A second dose was given just after the stroke surgery to maintain HG through 90 minutes of ischemia. Blood glucose was measured from the tail vein using a glucometer (Freestyle, Alameda, CA). Plasma osmolality was measured using advanced micro-osmometer, model 3300 (Advanced instruments, INC., Norwood, MA).

Study 2. To determine the effect of tPA and method of reperfusion in hyperglycemic stroke, control (NG) or mild HG (140 – 200 mg/dl) male Wistar rats were subjected to either suture or thromboembolic occlusion of the MCA. Animals were randomized to receive either tPA (Cathflo Activase (Alteplase), Genentech) or vehicle (water for injection) of equal volume. tPA (1mg/Kg) was intravenously infused over 20 minutes through the jugular vein 2h after induction of ischemia to rats with either suture or thromboembolic occlusion.

Preparation of Blood Clots

The method of clot preparation is the same as we previously reported [21, 22] but with minor modifications for rats to further increase the stability and strength of the freshly prepared blood clots. Briefly, arterial blood was withdrawn from a donor rat in a syringe

prefilled with human fibrinogen (10 mg/ml). The blood was immediately pushed into 20 cm polyethylene (PE)-50 tubes. The tubes were kept for 4-6 h at room temperature and then at 4ºC for 24 h. The PE-50 tubes containing the clots were cut into 5 cm long pieces and the formed clots were pushed out into a petri dish containing sterile water for injection and allowed to wash for 15 minutes. Blood clots were then transferred to a petri dish containing PBS and left for up to 6 h for retraction at room temperature and then washed with PE-10 tubes. A single piece (3-4 cm long) of clot was withdrawn in a PE-10 catheter and used to induce MCAO.

Stroke Surgery

Focal cerebral ischemia was performed as previously described [23]. Briefly, 90 min MCAO was performed under 2% isoflurane anesthesia followed by up to 24 h reperfusion. A midline cervical incision was made to expose the common carotid artery. The external carotid artery (ECA) was separated, ligated and cauterized. An arteriotomy was performed on the ECA stump. A rounded-tip 3-0 monofilament nylon suture (prepared carefully under microscope with high magnification power to ensure uniformity) was inserted into the ECA stump and advanced through the internal carotid artery to occlude the origin of MCA. The occlusion suture was secured with one-silk suture at the stump of ECA, and the incision was closed. After 90 min, animals were re-anesthetized, and the occlusion suture was removed to allow reperfusion. For the embolic model, the surgical procedure remained the same as in suture model except that instead of the monofilament suture, a PE-10 catheter

containing the clot was inserted through the ECA stump to deliver the clot to the origin of the MCA and produce occlusion. The clot was gently injected with 100 µL sterile saline. Scanning laser Doppler (Pim-3, Perimed, ST) was used to confirm a similar degree of drop in CBF among groups. The percent drop in CBF after stroke was determined by comparing to baseline [24]. To monitor the ability of tPA to resolve the blood clots and restore blood flow, CBF was measured 30, 60 and 120 minutes after tPA administration in animals subjected to thromboembolic occlusion. Animals with reduction in CBF less than 40% or more than 75% from baseline are excluded.

Evaluation of Infarct Size, Edema, HT and Hemoglobin (Hb) Content

At 24 h, and just before sacrifice, animals were put into deep sleep using isoflurane and subjected to intracardiac perfusion with ice cold PBS to flush out blood from cerebral vessels. Brains were isolated and sliced into 7 coronal sections marked A to G (+6.7mm to -8.8mm from the bregma). The infarct size was measured after staining the brain slices with 2,3,5-triphenyl tetrazolium chloride (TTC) as previously described [23]. Edema was calculated as a percent (%) increase in the ischemic hemisphere vs. the contralateral hemisphere. The stained brain sections are then separated into ipsilateral and contralateral hemispheres, snap frozen and kept at -80ºC for biochemical assays. Following brain homogenization, Hb content was measured with Quanti-Chrom kit (BioAssay Systems, Hayward, CA), 2007) and reported as excess hemoglobin (Hb, mg/g protein) in the ischemic hemisphere normalized to sham animals. A blinded investigator scored

macroscopic HT in brain slices B to E using a four-point rubric (0- No hemorrhage; 1- Dispersed individual petechiae; 2- Confluent petechiae; 3- Small diffuse hemorrhage or hematoma; 4- Large diffuse hemorrhage or hematoma) and the total score for each animal was reported [25].

Evaluation of Neurobehavioral and Functional Outcomes

Neurobehavioral tests were assessed, recorded and scored in a blinded fashion as previously described [26]. Briefly, animals were handled for 5–7 days prior to behavior testing in rooms where behavior testing is to be carried out. Neurobehavioral evaluation involved Bederson's score, beam walk and grip strength tests. Behavior testing was performed before stroke and at 24 h reperfusion, just before sacrifice. Bederson's score for each rat was obtained by using 3 parameters which include (a) observation of no circling scored as 2, partial circling scored as 1, continuous circling scored as 0, (b) hindlimb retraction and (c) forelimb flexion scored as 1 or 0 according to animal’s ability to immediately replace the limb upon pulling or not, respectively. The resistance to push is also measured and scored as 1 or 0, depending on whether the animal is able to resist pushing or not. Maximum score of 7 is given to a normal rat. Beam walking ability is graded based on a 7-point scale method previously described [25]. Total composite neurological score out of 14 composed of sum of the Bederson's score and the scores obtained from the beam-walking test. Forelimb grip strength was measured with a standard grip strength meter (Columbus Instrument, Columbus, OH).

Data Analysis

Data are presented as mean ± SD. Data were examined for outlying observations and nonparametric analyses were used when needed. Area under the curve was determined for blood glucose from baseline to 24 hours post-ischemia and for CBF as a percent of baseline using NCSS 2007 (NCSS, LLC, Kaysville, UT). The rCBF values post-reperfusion were compared to pre-reperfusion values using a paired t-test. The effects of mild and severe hyperglycemia were determined using a one-way ANOVA with three groups (Control, Mild HG and Severe HG). An exact Kruskal-Wallis test was used to analyze HT due to outlying values in the HG groups. The effect of hyperglycemia and tPA treatment was assessed using a 2 HG (no vs. yes) by 2 tPA (no vs. yes) ANOVA where a significant interaction would indicate a differential effect of tPA on stroke outcomes dependent on HG status. SAS© 9.3 (SAS, Inc., Cary, NC) was used for all analyses. Statistical significance was determined at alpha<0.05 and a Tukey's post-hoc test was used to compare means from significant ANOVAs.

Results

The Impact of the Severity of Hyperglycemia on Neurovascular Injury and Functional Outcomes

Administration of 30% and 60% glucose solutions achieved graded blood glucose levels of 140–200 and 260–350 mg/dl (Fig. 2.1A), respectively. Plasma osmolality was 309±3.2, 310±1.9 and 313±2 mosmol for the control, mild HG and severe HG groups, respectively. There was no significant difference in plasma osmolality between groups. The mortality was 10-15% in each group. The drop in CBF after occlusion was almost the same among the different animal groups (Fig. 2.1B). Mild elevation in blood glucose (140–200 mg/dl) did not increase the infarct size when compared to control animals. However, severe HG (240–350 mg/dl) caused a significant increase in the infarct size (Fig. 2.1C).

Edema in the brain, HT and Hb content were used to assess the vascular injury. Although mild elevation in BG did not increase the infarct size, it significantly increased bleeding in the brain when compared to control animals. All levels of HG, whether mild or severe, significantly increased the edema in the brain, macroscopic HT, and Hb content in the ischemic hemispheres (Fig. 2.2A-C).

Mild and severe levels of acute HG significantly reduced the grip strength and exacerbated the neurological deficits when compared to control normoglycemic animals (Fig. 2.3A, B).

The Impact of the Combination of Acute HG and tPA in Suture and Thromboembolic Models of Hyperglycemic Reperfusion on Neurovascular Injury and Functional Outcomes

Since mild HG exacerbated HT and edema and worsened functional outcomes, the next set of studies used this level of HG to assess the impact of tPA and the method of reperfusion. Administration of tPA did not affect blood glucose levels in different stroke models (data not shown). There was no difference in percent drop in CBF measured immediately either before reperfusion in the suture group or before tPA administration in the embolic group (Fig. 2.4A, B). In the thromboembolic model, laser Doppler monitoring showed that the first remarkable reperfusion was 30 to 60 minutes after tPA administration and achieved only 40 to 60% increase in CBF and this level persisted after tPA reperfusion up till sacrifice (Fig. 2.4B).

tPA treatment did not affect the infarct size in either normoglycemic or hyperglycemic animals when compared to their non-treated counterparts, whether these animals were subjected to suture or embolic occlusion (Fig. 2.5A, B). The administration of tPA slightly but not significantly increased the HT in control animals. However, the combination of HG with tPA significantly exacerbated the cerebrovascular injury and HT in the brain in both suture and embolic occlusion models (Fig. 2.6A, B). The interaction between tPA and HG led to a synergistic effect in exacerbating HT in animals subjected to embolic stroke (interaction p value = 0.029, Fig. 2.6B). The intracerebral bleeding was further confirmed by assessing the Hb content in the ischemic hemispheres. tPA administration increased the Hb content in the ischemic hemispheres in both normoglycemic and hyperglycemic animals irrespective of the method of reperfusion (Fig.

2.6C, D). tPA administration also increased edema in both suture and thromboembolic occlusion animal models (Fig. 2.6E, F).

tPA significantly reduced the grip strength and neurological scores resulting in worse functional outcomes in normoglycemic and hyperglycemic animals compared to untreated animals in both models of reperfusion. However, the combination of tPA and HG significantly exacerbated the neurological deficits compared to each alone (Fig. 2.7A-D). The neurological deterioration showed a similar pattern in both suture and embolic occlusion models (Fig. 2.7A-D).

Discussion

One goal of this preclinical study was to bridge the critical gap between clinical observations and experimental studies in acute hyperglycemic stroke with regard to blood glucose levels and use of tPA. Accordingly, this study was designed to address the following points: 1.The impact of the severity of acute HG on stroke outcomes using clinically relevant blood glucose levels; 2. The impact of the interaction between acute HG and tPA on neurovascular injury and functional outcomes in different experimental stroke models that mimic the clinical scenario; and 3. Establishing a novel approach for studying the hyperglycemia reperfusion injury in an embolic stroke model with human tPA dose.

Clinically, HG is an independent predictor of poor functional outcomes for AIS patients. The TOAST trial showed that higher admission blood glucose was associated with worse clinical outcomes in patients with non-lacunar stroke [27]. Admission HG was

shown to be an independent predictor of poor neurological outcomes and increased rate of symptomatic intracranial hemorrhage (ICH) in the NINDS and ATLANTIS trials [28, 29]. The ECASS II trial also showed that HG persisting for 24 h or more is an independent predictor of poor clinical outcomes, ICH and death in AIS patients [30, 31]. Moreover, HG aggravates tPA induced cerebral hemorrhage in AIS patients [14, 15, 29]. Accordingly, several clinical trials were conducted to examine the safety, feasibility and efficacy of lowering blood glucose for hyperglycemic AIS patients. The Treatment of HG in Ischemic Stroke (THIS) and the Glucose Regulation in Stroke Patients (GRASP) trials, showed that, in these cohorts, blood glucose levels range between 160 – 260 mg/dl at admission and they targeted blood glucose levels of less than 130 and 110 mg/dl, respectively [32, 33]. Based on the results of these feasibility studies, the Stroke Hyperglycemia Insulin Network Effort (SHINE) trial is currently recruiting patients to examine the efficacy and safety of blood glucose lowering in AIS patients targeting blood glucose of 80 – 130 mg/dL [34]. However, evidence from clinical trials including The Normoglycemia in Intensive Care Evaluation-Survival Using Glucose Algorithm Regulation (NICE-SUGAR) and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trials showed that intensive blood glucose lowering was associated with worse outcome and increased mortality [35, 36]. The incidence of hypoglycemia (<60 mg/dL) was the major cause of adverse events and increased mortality. Blood glucose target <180 mg/dL resulted in less mortality than did a target of 80-108 mg/dL [35]. The UK Glucose Insulin in Stroke Trial (GIST-UK) showed that tight glycemic control did neither improve the mortality nor the neurologic impairment at 90 days in hyperglycemic AIS patients but the study was terminated early due to slow

recruitment [37]. These past studies demonstrate the challenges in the field and emphasizes the importance of the cautious and conservative control of blood glucose in AIS patients.

While past preclinical studies provided very valuable information on the impact and mechanisms by which HG worsens stroke outcomes, they used hyperglycemia models with much higher blood glucose levels (350 – 500 mg/dL) than those observed in patients [7, 8, 19, 38]. To address this gap, in the current study we used graded blood glucose levels that mimic the clinical scenario. In this study, we found that mild elevation in blood glucose increased edema and HT without increasing the infarct size. This reflects the sensitivity and the vulnerability of the vasculature to acute yet subtle changes in blood glucose and also reflects the role the vasculature plays in contributing to worse functional outcomes when there is no further neuronal injury.

Our findings provide evidence that the severity of acute HG is a determinant of functional outcome. Severe HG significantly increased the infarct size compared to control and mild HG groups and this was associated with worse functional outcomes. The major cause of this damage may be attributed to the increase in generation of reactive oxygen species (superoxide and peroxynitrite) and inflammatory cytokines (TNF alpha and interleukins) [1, 4, 6, 8, 39, 40]. Another possibility may be increased osmolality. Bhardwaj et al showed that continuous infusion of hypertonic saline for 22 h increased plasma osmolality and this was associated with an increase in the infarct volume after transient focal ischemia in rats [41]. However, we did not observe a significant difference in plasma osmolality in the current study which may be due to the small bolus dose of glucose solution that we used to induce acute HG.

As recently reviewed, almost all the previous experimental studies in acute hyperglycemic stroke research used the suture occlusion model to induce stroke [7]. However, this model allows abrupt reperfusion and prompt restoration of CBF to the ischemic tissue, which may exacerbate the damage. Moreover, it does not mimic what happens in ischemic stroke patients where the ischemia is due to occlusion of cerebral vessels by blood clots, which further increases risk of microemboli formation and entrapment. In this translational study, we used both suture and thromboembolic occlusion models to recapitulate the clinical condition. Our findings show that the infarct size is greater in the embolic model (most likely due to longer occlusion). However, both models cause a similar degree of HT and neurological deficit in acute HG, in spite of the shorter occlusion time achieved with the suture model. This is most probably due to the prompt reperfusion that takes place in the suture model and flushes the ischemic tissue with a massive and sudden flow of reactive oxygen species and inflammatory cytokines. We recently reported that embolic stroke causes less HT in a diabetic model as compared to suture occlusion and suggested that gradual reperfusion that occurs in embolic stroke may have lessened the [39]injury [22]. Current results raise the possibility that under diabetic conditions compensatory changes may limit the vascular injury.

Although the interaction between HG and tPA has been shown to be detrimental to AIS patients, very few experimental studies were conducted to study this. The limited experimental studies with the embolic model used streptozotocin-induced diabetes, not acute hyperglycemia, with very high blood glucose levels. Based on early studies which reported differences in rat's fibrinolytic system [42], these studies used a 10-fold higher dose of tPA (10 mg/kg) than that what is used in ischemic stroke patients and reported

increased intracerebral hemorrhage [16-19]. Recently, several groups used low dose of tPA in combination with other drugs in order to improve the outcomes and at the same time avoid the adverse effects of high dose tPA. The combination of low dose tPA (2.5 mg/kg) with annexin A2 (5 mg/kg) in rats subjected to embolic stroke, significantly improved CBF, reduced brain infarction and HT [43]. A higher dosage regimen of 5 mg/kg tPA/10 mg/kg annexin A2 was able to improve the long term neurological outcomes 1 month post stroke [44]. These studies were conducted in normal animals. In the current study we used a low dose of tPA (1 mg/kg), which is almost the human dose. We hypothesized that tPA treatment will reduce infarct and improve outcomes in the control (NG) animals and expected greater HT and neurological deficits in the HG group. However, we didn't see a significant difference in infarct size in animals receiving tPA as compared to vehicle group. This is most probably because the tPA-induced reperfusion was not sufficient to limit the infarct expansion even in the control group. Prolonged monitoring of CBF demonstrated that the low dose tPA given at 2 h post-occlusion was not able to fully restore CBF which may have contributed to this finding. Past studies reported lower [45] or similar infarct volumes [46-48] as we observed in this study. In the current study, tPA increased bleeding and worsened functional outcome. Lapergue et al also reported increased HT after tPA treatment in an embolic model but they did not report any functional outcomes [47]. Several other studies showed increased BBB permeability and there was no effect of tPA on functional outcomes as compared to vehicle treated groups [46, 49]. While we do not have a full explanation of poor outcomes in the control group treated with tPA, there is a significant increase in bleeding which may be responsible for poor outcomes. We also

measured behavioral outcomes at 24 h while injury is still evolving. Late time points may be needed to address this concern.

The rationale to use tPA in the suture model was to assess the effects of tPA on neuronal injury and HT irrespective of its recanalization capability. Our results show that infarct size is similar between vehicle and tPA-treated animals suggesting that "low dose" tPA does not have additional effects on cell death (core formation) as previously reported [45, 47]. These past studies either did not report HT and functional outcomes or reported HT without functional outcomes. In the current study, we show that tPA increased HT and worsened the outcome emphasizing the role of vascular injury on functional outcomes. There are also studies which showed reduction in infarct size with tPA. Berny-Lang et al showed that tPA (2.5 mg/kg) given early during MCAO (15 minutes after inducing ischemia), reduced infarct size in mice but not rats with suture occlusion. However, they did not report the infarct size when they gave tPA 2 hours after reperfusion [50]. Kilic et al also reported reduction in ischemic injury in mice when treated with tPA 10 mg/kg during early MCAO [51]. These differences in outcomes may be due to the difference in dose and timing of tPA treatment.

Conclusion and Limitations

Up to date, tPA remains to be the only therapeutic option for AIS patients. Given that almost 50% of stroke patients present with HG, which aggravates the tPA-induced cerebral hemorrhage, there is a pressing need for more preclinical studies in which important

clinical questions can be addressed. For example, clinical studies suggest that women benefit more from tPA therapy as compared to men [52]. While the use of only healthy, male young animals and assessment of only short term outcomes remain to be limitations of the current study, to the best of our knowledge, this is the first study investigating the interaction between tPA and acute HG in a clinically relevant model of embolic stroke. This translational study is also the first to compare the effects of acute HG in different models of reperfusion injury.

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Compliance with Ethics Requirements

All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of Interest

Sherif Hafez declares that he has no conflict of interest.

Md Nasrul Hoda declares that he has no conflict of interest.

Xinyue Guo declares that she has no conflict of interest.

Maribeth H. Johnson declares that she has no conflict of interest.

Susan C. Fagan declares that she has no conflict of interest.

Adviye Ergul declares that she has no conflict of interest.

Figure Legends

Figure 2.1. A) The hyperglycemia model. Administration of 30% and 60% glucose solutions successfully achieved blood glucose levels of 140-200 (mild HG) and 240-350 (severe HG) mg/dl, respectively. Acute HG was maintained through 90 minutes of ischemia. B) The percent drop in cerebral blood flow from baseline was almost the same among the different animal groups. C) Mild HG did not affect the infarct size, however, severe HG significantly increased the infarct size when compared to control and mild HG animals. N= 5-7/group. *$p<0.05$ vs control, #$p<0.05$ vs mild HG.

Figure 2.1. Hyperglycemia model, CBF and Infarct size.

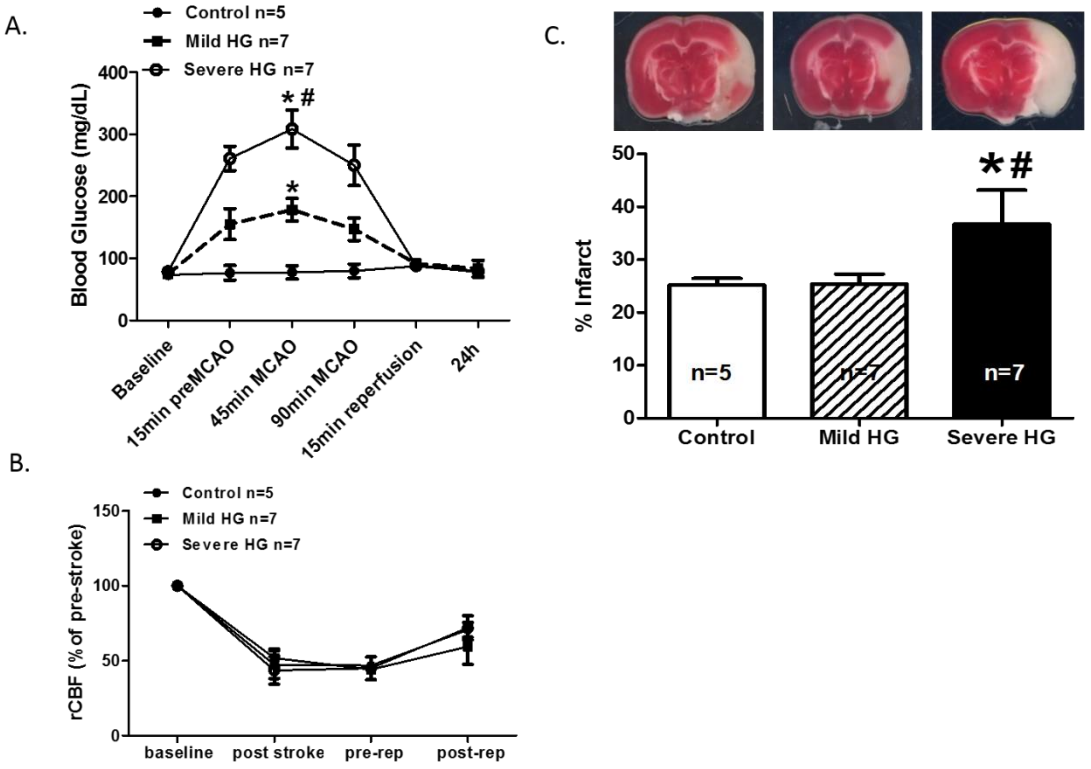


Figure 2.2. All levels of HG whether mild or severe significantly exacerbated the cerebrovascular injury in the brain. A) Representative images of HT patterns are shown on top. HT index, shown at the bottom as a measure of macroscopic HT occurrence, demonstrates increased HT in hyperglycemic animals. B) HG significantly increased Hb content in the ischemic hemisphere which is used as a measure of the severity of bleeding. C) HG also increased edema in the brain. *$p<0.05$ vs control.

Figure 2.2. HT, Hb and Edema.

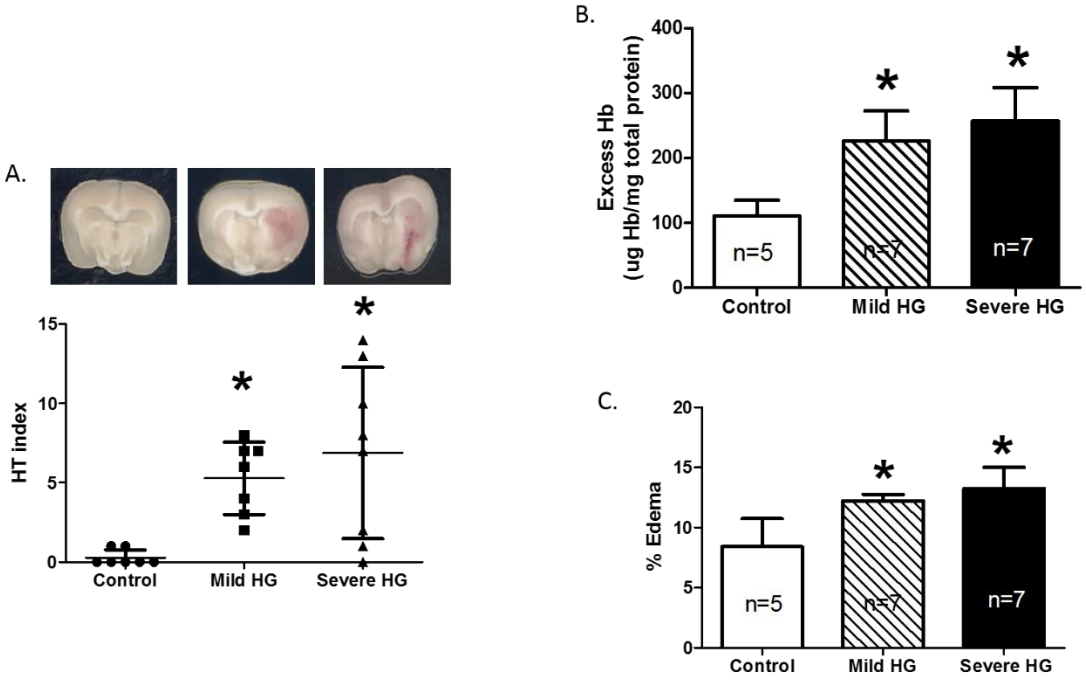


Figure 2.3. Mild HG significantly exacerbated the neurological deficits and worsened the functional outcomes as indicated by A) composite score and B) grip strength. Severe HG caused even worse deterioration compared to mild HG. Lower composite score indicates greater neurological deficits. *$p<0.05$ vs control, #$p<0.05$ vs mild HG.

Figure 2.3. Functional outcomes.

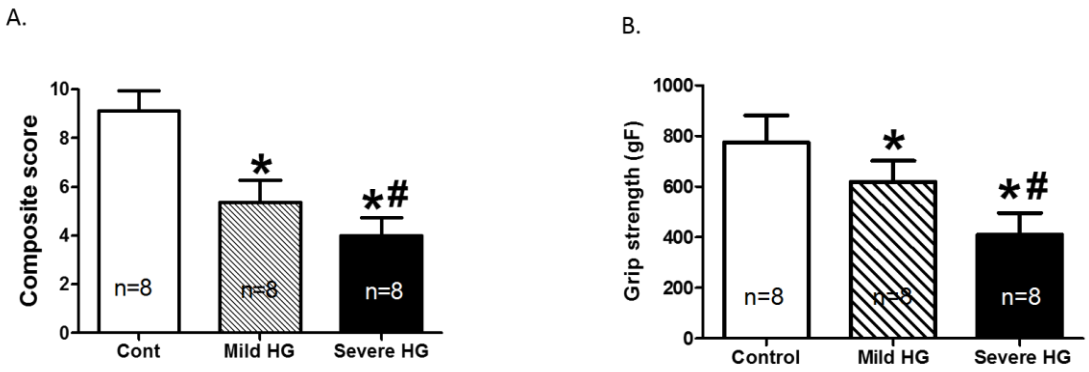


Figure 2.4. A) The percent drop in CBF at 90 minutes occlusion (just before reperfusion) is almost the same among the different animal groups subjected to suture occlusion. B) The drop in CBF at 2h in rats subjected to thromboembolic occlusion was almost the same among tPA treated and non-treated groups. The use of low dose tPA (1 mg/kg) was able to slightly and gradually restore the blood flow through a period of 2 h. *$p<0.05$ vs pre-tPA and vehicle.

Figure 2.4. CBF: Suture and Embolic.

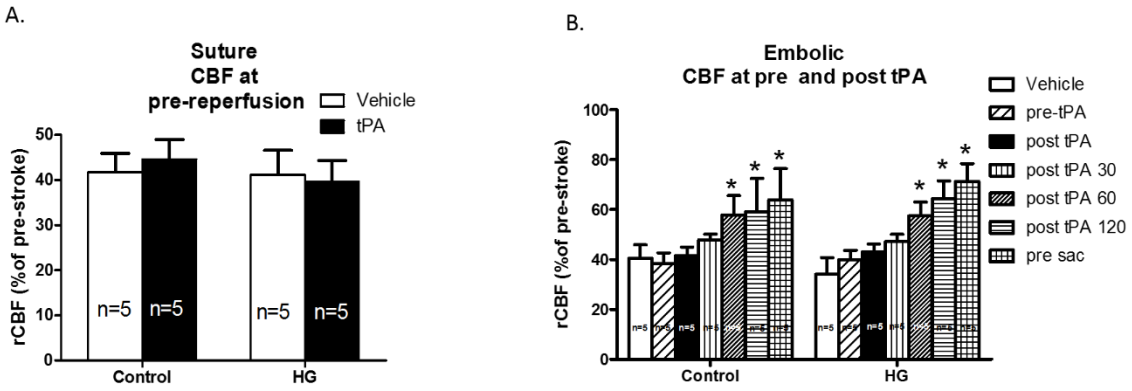


Figure 2.5. Administration of tPA did not affect the infarct size in different animal groups subjected to either A) suture or b) thromboembolic occlusion. However, due to prolonged occlusion, embolic stroked animals developed larger infarcts than those with suture occlusion.

Figure 2.5. Infarct size: Suture and Embolic.

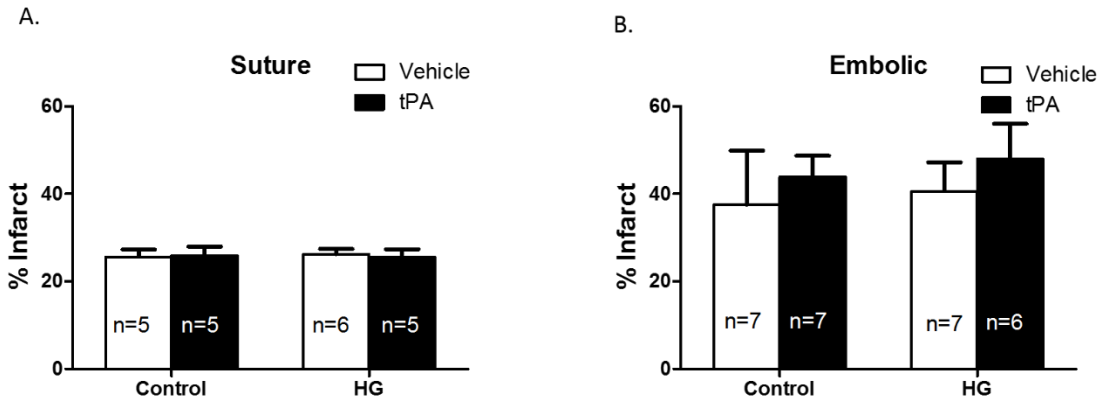


Figure 2.6. HG and tPA augment vascular injury. A) Mild acute HG significantly increased the macroscopic HT in animals subjected to suture occlusion compared to control. B) In animals with embolic stroke, mild HG showed a trend (p value= 0.075 vs control) but not a significant increase in macroscopic HT when compared to control. The combination of HG and tPA exacerbated the injury and showed a significant increase in HT more than each alone in both reperfusion models. The interaction between HG and tPA potentiated the HT in animals subjected to embolic stroke (λp=0.029, interaction between HG and tPA, Fig. 6B). Mild HG and tPA individually have significantly increased the Hb content in the ischemic hemispheres compared to control animals in both C) suture and D) embolic occlusion models. The combination of HG and tPA worsened the bleeding and significantly increased the Hb content when compared to each of them alone, independent of the model of reperfusion. Mild HG significantly increased edema in the brains of animals subjected to either E) suture or F) embolic occlusion. The combination of HG and tPA significantly increased edema when compared to each alone in animals with embolic (6F) but not suture occlusion (6E). *p<0.05 vs groups receiving vehicle (no tPA) either control or HG, #p<0.05 vs control (NG) receiving vehicle (no HG, no tPA), ¥p<0.05 vs control treated with tPA alone (no HG). In Fig. 6B, λp <0.05, and (λ) indicates the significant interaction between HG and tPA that significantly potentiated the HT in rats subjected to embolic stroke.

Figure 2.6. HT, Hb and Edema: Suture and Embolic.

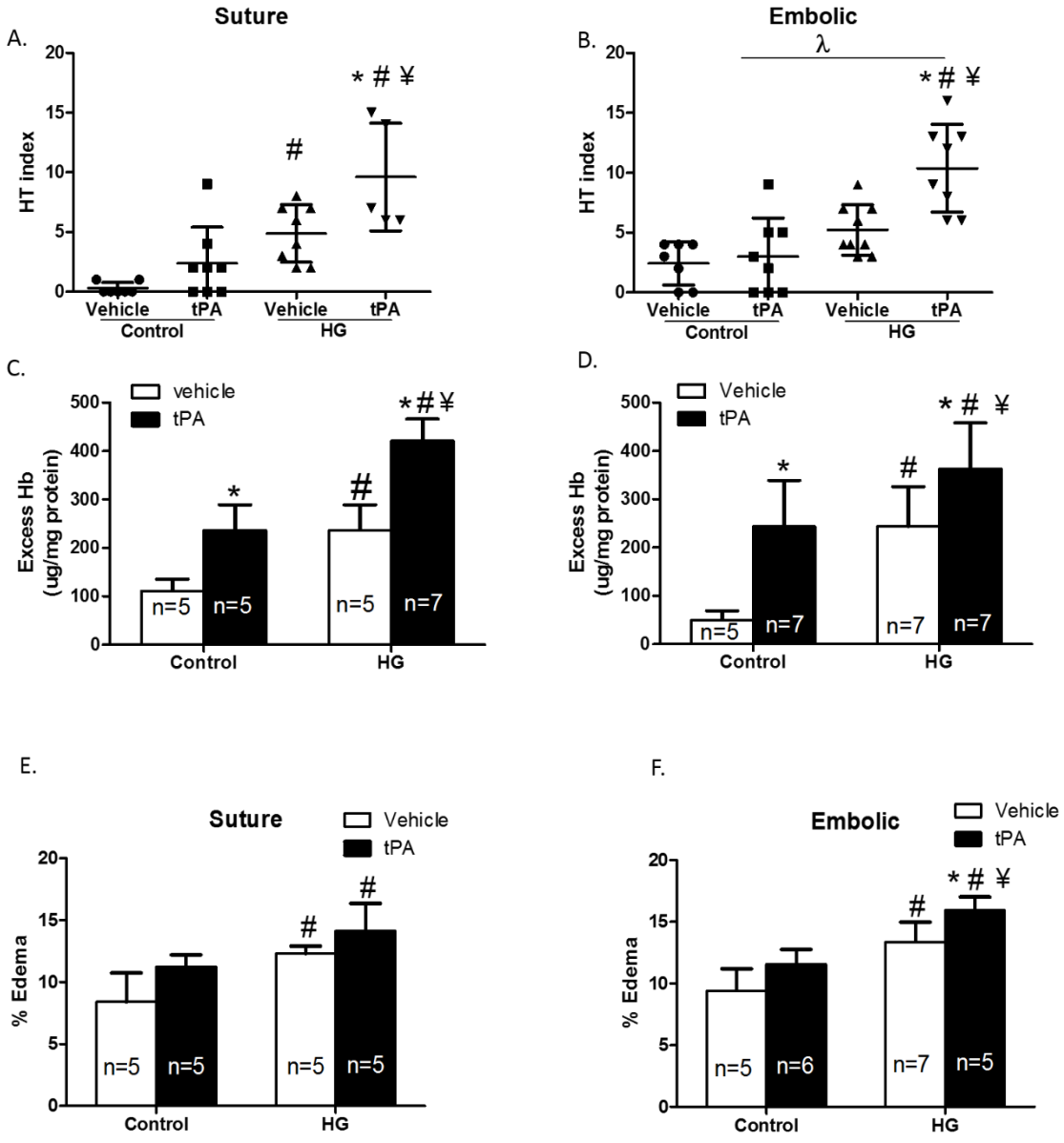
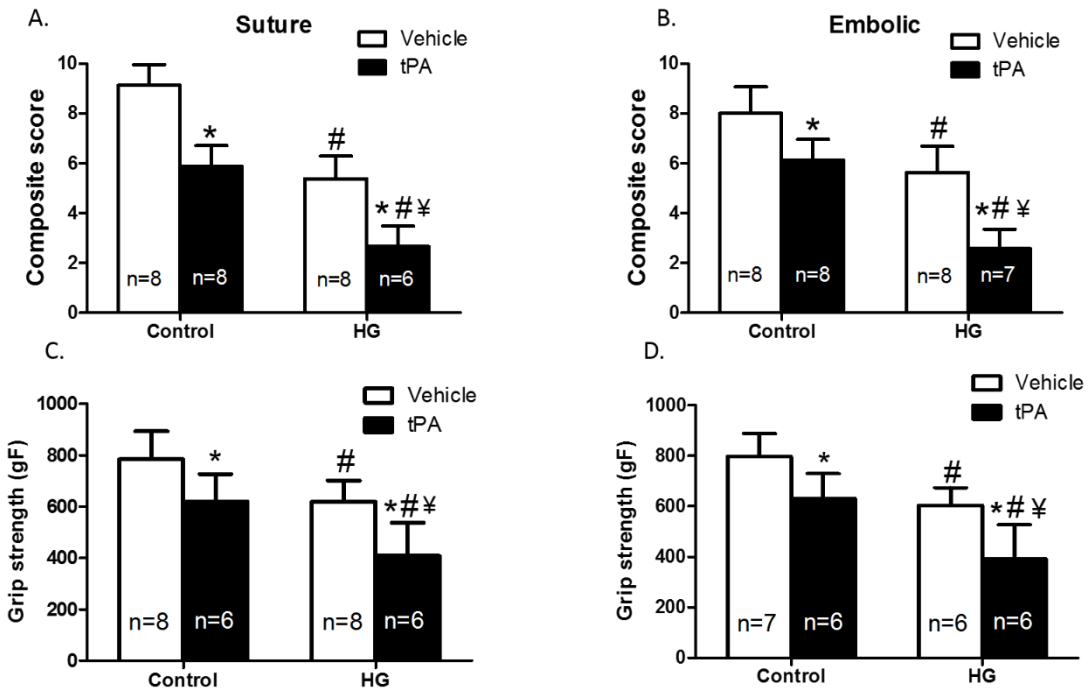


Figure 2.7. HG and tPA worsen functional outcomes. Both HG and tPA significantly worsened the neurological deficits and reduced the grip strength in suture (A and C) and embolic (B and D) occlusion models. The combination of HG and tPA further exacerbated the injury and worsened the outcome more than each alone in both models of reperfusion. *$p<0.05$ vs groups receiving vehicle (no tPA) either control or HG, #$p<0.05$ vs control receiving vehicle (no HG, no tPA), ¥$p<0.05$ vs control treated with tPA alone (no HG).

Figure 2.7. Functional outcomes: Suture and Embolic.



BIBLIOGRAPHY

1. Kruyt, N.D., et al., *Hyperglycemia in acute ischemic stroke: pathophysiology and clinical management.* Nat Rev Neurol, 2010. 6(3): p. 145-55.
2. Capes, S.E., et al., *Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview.* Stroke, 2001. 32(10): p. 2426-32.
3. Quast, M.J., et al., *Perfusion deficit parallels exacerbation of cerebral ischemia/reperfusion injury in hyperglycemic rats.* J Cereb Blood Flow Metab, 1997. 17(5): p. 553-9.
4. Yip, P.K., et al., *Effect of plasma glucose on infarct size in focal cerebral ischemia-reperfusion.* Neurology, 1991. 41(6): p. 899-905.
5. Suh, S.W., et al., *Glucose and NADPH oxidase drive neuronal superoxide formation in stroke.* Ann Neurol, 2008. 64(6): p. 654-63.
6. Wei, J., N.C. Huang, and M.J. Quast, *Hydroxyl radical formation in hyperglycemic rats during middle cerebral artery occlusion/reperfusion.* Free Radic Biol Med, 1997. 23(7): p. 986-95.
7. Hafez, S., et al., *Hyperglycemia, Acute Ischemic Stroke, and Thrombolytic Therapy.* Transl Stroke Res, 2014.
8. Kamada, H., et al., *Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction.* Stroke, 2007. 38(3): p. 1044-9.
9. Elgebaly, M.M., et al., *Neurovascular injury in acute hyperglycemia and diabetes: A comparative analysis in experimental stroke.* Transl Stroke Res, 2011. 2(3): p. 391-398.
10. Cipolla, M.J., Q. Huang, and J.G. Sweet, *Inhibition of protein kinase Cbeta reverses increased blood-brain barrier permeability during hyperglycemic stroke and prevents edema formation in vivo.* Stroke, 2011. 42(11): p. 3252-7.
11. Xing, Y., et al., *Effects of deferoxamine on brain injury after transient focal cerebral ischemia in rats with hyperglycemia.* Brain Res, 2009. 1291: p. 113-21.
12. Kumari, R., et al., *The PPAR-gamma agonist, darglitazone, restores acute inflammatory responses to cerebral hypoxia-ischemia in the diabetic ob/ob mouse.* J Cereb Blood Flow Metab, 2010. 30(2): p. 352-60.
13. Lees, K.R., et al., *Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials.* Lancet, 2010. 375(9727): p. 1695-703.
14. Poppe, A.Y., et al., *Admission hyperglycemia predicts a worse outcome in stroke patients treated with intravenous thrombolysis.* Diabetes Care, 2009. 32(4): p. 617-22.
15. Alvarez-Sabin, J., et al., *Effects of admission hyperglycemia on stroke outcome in reperfused tissue plasminogen activator--treated patients.* Stroke, 2003. 34(5): p. 1235-41.
16. Ning, R., et al., *Tissue plasminogen activator treatment of stroke in type-1 diabetes rats.* Neuroscience, 2012. 222: p. 326-32.

17. Fan, X., E.H. Lo, and X. Wang, *Effects of minocycline plus tissue plasminogen activator combination therapy after focal embolic stroke in type 1 diabetic rats.* Stroke, 2013. 44(3): p. 745-52.
18. Fan, X., et al., *Early insulin glycemic control combined with tPA thrombolysis reduces acute brain tissue damages in a focal embolic stroke model of diabetic rats.* Stroke, 2013. 44(1): p. 255-9.
19. Fan, X., et al., *A rat model of studying tissue-type plasminogen activator thrombolysis in ischemic stroke with diabetes.* Stroke, 2012. 43(2): p. 567-70.
20. Won, S.J., et al., *Hyperglycemia promotes tissue plasminogen activator-induced hemorrhage by Increasing superoxide production.* Ann Neurol, 2011. 70(4): p. 583-90.
21. Hoda, M.N., et al., *Sex-independent neuroprotection with minocycline after experimental thromboembolic stroke.* Exp Transl Stroke Med, 2011. 3(1): p. 16.
22. Li, W., et al., *Comparative analysis of the neurovascular injury and functional outcomes in experimental stroke models in diabetic Goto-Kakizaki rats.* Brain Res, 2013. 1541: p. 106-14.
23. Ergul, A., et al., *Increased hemorrhagic transformation and altered infarct size and localization after experimental stroke in a rat model type 2 diabetes.* BMC Neurol, 2007. 7: p. 33.
24. Guan, W., et al., *Acute Treatment with Candesartan Reduces Early Injury After Permanent Middle Cerebral Artery Occlusion.* Translational Stroke Research, 2011: p. 1-7.
25. Kelly-Cobbs, A.I., et al., *Targets of vascular protection in acute ischemic stroke differ in type 2 diabetes.* Am J Physiol Heart Circ Physiol, 2013. 304(6): p. H806-15.
26. Prakash, R., et al., *Vascularization Pattern After Ischemic Stroke is Different in Control Versus Diabetic Rats: Relevance to Stroke Recovery.* Stroke, 2013.
27. Bruno, A., et al., *Acute blood glucose level and outcome from ischemic stroke. Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators.* Neurology, 1999. 52(2): p. 280-4.
28. Clark, W.M., et al., *The rtPA (alteplase) 0- to 6-hour acute stroke trial, part A (A0276g) : results of a double-blind, placebo-controlled, multicenter study. Thromblytic therapy in acute ischemic stroke study investigators.* Stroke, 2000. 31(4): p. 811-6.
29. Bruno, A., et al., *Admission glucose level and clinical outcomes in the NINDS rt-PA Stroke Trial.* Neurology, 2002. 59(5): p. 669-74.
30. Yong, M. and M. Kaste, *Dynamic of hyperglycemia as a predictor of stroke outcome in the ECASS-II trial.* Stroke, 2008. 39(10): p. 2749-55.
31. Piironen, K., et al., *Glucose and acute stroke: evidence for an interlude.* Stroke, 2012. 43(3): p. 898-902.
32. Bruno, A., et al., *Treatment of hyperglycemia in ischemic stroke (THIS): a randomized pilot trial.* Stroke, 2008. 39(2): p. 384-9.
33. Johnston, K.C., et al., *Glucose Regulation in Acute Stroke Patients (GRASP) trial: a randomized pilot trial.* Stroke, 2009. 40(12): p. 3804-9.

34. Bruno, A., et al., *The Stroke Hyperglycemia Insulin Network Effort (SHINE) trial protocol: a randomized, blinded, efficacy trial of standard vs. intensive hyperglycemia management in acute stroke.* Int J Stroke, 2014. 9(2): p. 246-51.
35. Finfer, S., et al., *Intensive versus conventional glucose control in critically ill patients.* N Engl J Med, 2009. 360(13): p. 1283-97.
36. Gerstein, H.C., et al., *Effects of intensive glucose lowering in type 2 diabetes.* N Engl J Med, 2008. 358(24): p. 2545-59.
37. Gray, C.S., et al., *Glucose-potassium-insulin infusions in the management of post-stroke hyperglycaemia: the UK Glucose Insulin in Stroke Trial (GIST-UK).* Lancet Neurol, 2007. 6(5): p. 397-406.
38. Siemkowicz, E., A.J. Hansen, and A. Gjedde, *Hyperglycemic ischemia of rat brain: the effect of post-ischemic insulin on metabolic rate.* Brain Res, 1982. 243(2): p. 386-90.
39. Bemeur, C., L. Ste-Marie, and J. Montgomery, *Increased oxidative stress during hyperglycemic cerebral ischemia.* Neurochem Int, 2007. 50(7-8): p. 890-904.
40. Ste-Marie, L., et al., *Immunohistochemical detection of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase following hyperglycemic focal cerebral ischemia.* Brain Res, 2001. 918(1-2): p. 10-9.
41. Bhardwaj, A., et al., *Hypertonic saline worsens infarct volume after transient focal ischemia in rats.* Stroke, 2000. 31(7): p. 1694-701.
42. Korninger, C. and D. Collen, *Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various animal species in vitro.* Thromb Haemost, 1981. 46(2): p. 561-5.
43. Zhu, H., et al., *Annexin A2 combined with low-dose tPA improves thrombolytic therapy in a rat model of focal embolic stroke.* J Cereb Blood Flow Metab, 2010. 30(6): p. 1137-46.
44. Wang, X., et al., *Effects of tissue plasminogen activator and annexin A2 combination therapy on long-term neurological outcomes of rat focal embolic stroke.* Stroke, 2014. 45(2): p. 619-22.
45. Meng, W., et al., *Effects of tissue type plasminogen activator in embolic versus mechanical models of focal cerebral ischemia in rats.* J Cereb Blood Flow Metab, 1999. 19(12): p. 1316-21.
46. Zhang, L., et al., *Combination treatment with N-acetyl-seryl-aspartyl-lysyl-proline and tissue plasminogen activator provides potent neuroprotection in rats after stroke.* Stroke, 2014. 45(4): p. 1108-14.
47. Lapergue, B., et al., *High-density lipoprotein-based therapy reduces the hemorrhagic complications associated with tissue plasminogen activator treatment in experimental stroke.* Stroke, 2013. 44(3): p. 699-707.
48. Shehadah, A., et al., *Combination treatment with low-dose Niaspan and tissue plasminogen activator provides neuroprotection after embolic stroke in rats.* J Neurol Sci, 2011. 309(1-2): p. 96-101.
49. Zhang, L., et al., *Combination treatment with VELCADE and low-dose tissue plasminogen activator provides potent neuroprotection in aged rats after embolic focal ischemia.* Stroke, 2010. 41(5): p. 1001-7.

50. Berny-Lang, M.A., et al., *Thrombin mutant W215A/E217A treatment improves neurological outcome and reduces cerebral infarct size in a mouse model of ischemic stroke.* Stroke, 2011. 42(6): p. 1736-41.
51. Kilic, E., D.M. Hermann, and K.A. Hossmann, *Recombinant tissue plasminogen activator reduces infarct size after reversible thread occlusion of middle cerebral artery in mice.* Neuroreport, 1999. 10(1): p. 107-11.
52. Kent, D.M., et al., *Sex-based differences in response to recombinant tissue plasminogen activator in acute ischemic stroke: a pooled analysis of randomized clinical trials.* Stroke, 2005. 36(1): p. 62-5.

CHAPTER 3

MATRIX METALLOPROTEASE 3 EXACERBATES HEMORRHAGIC TRANSFORMATION AND WORSENS FUNCTIONAL OUTCOMES IN HYPERGLYCEMIC STROKE[2]

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MATRIX METALLOPROTEASE 3 EXACERBATES HEMORRHAGIC TRANSFORMATION AND WORSENS FUNCTIONAL OUTCOMES IN HYPERGLYCEMIC STROKE

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Abstract

Background and Purpose. Acute hyperglycemia was shown to worsen the clinical outcomes and exacerbates the cerebral hemorrhage after stroke. The mediators of hemorrhagic transformation (HT) in hyperglycemic stroke are not fully understood. Matrix metalloproteinase 3 (MMP3) was shown to mediate the tissue plasminogen activator induced HT. However, the role of MMP3 in exacerbating HT and functional outcomes in hyperglycemic stroke remains unknown.

Methods. Control/normoglycemic and hyperglycemic (blood glucose: 140-200 mg/dl) male Wistar rats were subjected to middle cerebral artery occlusion (MCAO) for 90 minutes and up to 24 h reperfusion. MMP3 activity was measured in brain homogenate and cerebral macrovessels. MMP3 was inhibited pharmacologically (UK 356618, 15 mg/kg IV at reperfusuion) or knocked down by shRNA lentiviral particles. Neurovascular injury and functional outcomes were studied at 24 hours. Localization of MMP3 within the neurovascular unit after hyperglycemic stroke was demonstrated by immunohistochemistry.

Results. Hyperglycemia significantly increased MMP3 activity in the brain after stroke and this was associated with exacerbated HT and worsened functional outcomes. MMP3 inhibition significantly reduced HT and improved functional outcomes in hyperglycemic animals after stroke.

Conclusion. MMP3 plays a critical role in mediating cerebrovascular injury in hyperglycemic stroke. Our findings point out MMP3 as a potential therapeutic target in hyperglycemic stroke.

Introduction

Ischemic stroke is a leading cause of death and disability and the mechanisms involved in the neurovascular deterioration after stroke are multifactorial and highly complicated [1]. Hyperglycemia (HG) is one of the factors that worsens the neurovascular injury and is considered an independent predictor of poor clinical outcomes after stroke [2, 3]. Almost 50% of acute ischemic stroke (AIS) patients present with HG at time of admission to the hospital and half of these patients develop acute HG as a stress response without a previous history of diabetes. It was found that these patients with acute HG suffer the least favorable clinical outcomes when compared to normoglycemic or even diabetic patients [4].

A large body of evidence from experimental and preclinical studies supports that HG exacerbates the neurovascular injury and worsens the functional outcomes after stroke through increasing the infarct size, HT and disruption of blood brain barrier [5, 6]. Previously we have shown that even mild to moderate HG exacerbated the vascular injury and functional outcomes without increasing the infarct size [7, 8]. However, the underlying mechanisms contributing to increased vascular injury and HT in hyperglycemic stroke are not fully understood.

Matrix metalloprotease 3 (MMP3) belongs to a big family of zinc endopeptidases that can target and degrade a large number of extracellular matrix and tight junction proteins of the neurovascular unit [9, 10] and promotes neurodegeneration [11]. While MMP3 has been shown to contribute to tissue plasminogen activator (tPA)-induced HT [12, 13], its role in mediating the neurovascular injury in hyperglycemic stroke remains unclear. Thus, in this study we tested the hypothesis that hyperglycemia worsens stroke outcomes through

elevated MMP3 activity and that MMP3 inhibition would reduce HT and improve functional outcomes in hyperglycemic stroke.

Materials and Methods

Animal Models and Study Groups

The animals were housed at the Georgia Regents University animal care facility, which is approved by the American Association for Accreditation of Laboratory Animal Care. This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research and all protocols were approved by the institutional animal care and use committee. All the STAIR (Stroke Therapy Academic Industry Roundtable) and RIGOR recommendations and guidelines regarding randomization, blinding and statistical analysis were followed in this study [14, 15].

Study 1. Determine the effect of HG on MMP3 activity and localization within the neurovascular unit during the acute phase of ischemic stroke.

Male Wistar rats (Harlan Laboratories Inc., Indianapolis, IN) were assigned to 2 different groups: (1) Control/Normoglycemic (NG) (BG 90 – 120 mg/dl), and (2) Mild HG (BG 140 – 200 mg/dl). Animals were subjected to 90 minutes middle cerebral artery occlusion (MCAO) by the suture occlusion method and up to 24 hours reperfusion as previously described [8]. In all groups, animals with reduction in cerebral blood flow less than 40% from baseline were excluded. 30% glucose solution was used to achieve blood glucose levels of 140 - 200 mg/dl. Acute HG was achieved through intraperitoneal (IP) injection of 2 ml glucose solution 15 min before MCAO. A second dose was given just after the stroke surgery to maintain HG through 90 minutes of ischemia. At 24 h, brains and

macrovasculature (Circle of Willis vessels) were isolated and processed for MMP3 activity assay as well as immuno-localization studies.

Study 2. Determine the effect of MMP3 inhibition on reducing the HT and improving outcomes in hyperglycemic stroke.

Study 2.1. Pharmacological inhibition of MMP3.

Male Wistar rats (Harlan Laboratories Inc., Indianapolis, IN) were assigned to the following groups: (1) Control/NG (BG 90-120 mg/dl), (2) Mild HG (BG 140-200 mg/dL), (3) Mild HG + MMP3 inhibitor. In this study we used a potent and highly selective MMP3 inhibitor (UK 356618, Tocris Bioscience). The drug was intravenously injected through the jugular vein (15mg/kg) at reperfusion. The dose was determined according to a dose titration study and dose response curve to achieve an inhibition of MMP3 activity of 50% or more from non-treated animals (data not shown). Acute HG and stroke were induced as in Study 1 and neurovascular injury and functional outcomes were measured at 24 h.

Study 2.2. MMP3 knockdown in the brain via stereotaxic injection.

Male Wistar rats were anesthetized with isoflurane and immobilized on a stereotaxic device 2 weeks before MCAO. MMP3 shRNA lentiviral particles (5 µl of 1x 10^8 TU/ml, SMART choice lentiviral rat MMP3 shRNA, cat # SH-092769-02, GE Healthcare Dharmacon Inc., USA) or an empty vector (EV) was injected in both hemispheres over 10 min/side via a 30-gauge needle to the lateral ventricles. The stereotaxic coordinates were +0.9 mm anterior, ± 1.5 mm lateral, - 4 mm ventral relative to bregma. The study groups were (1) NG + EV, (2) HG + EV, (3) NG + MMP3 shRNA, (4) HG + MMP3 shRNA. MMP3 knockdown was confirmed by Western blot analysis.

Evaluation of Neurobehavioral and Functional Outcomes

At 24 h, neurobehavioral tests (Bederson's score, beam walk and grip strength tests) were assessed on a 14-point scale in a blinded fashion as previously described [8].

Evaluation of Infarct Size, Edema, HT and Hemoglobin (Hb) Content and Mortality

A blinded investigator scored macroscopic HT in brain slices B to E using a four-point rubric (0- No hemorrhage; 1- Dispersed individual petechiae; 2- Confluent petechiae; 3- Small diffuse hemorrhage or hematoma; 4- Large diffuse hemorrhage or hematoma) and the total score for each animal was reported. The infarct size was measured after staining the brain slices with 2,3,5-triphenyl tetrazolium chloride (TTC) as previously described.[8, 16] Edema was calculated as a percent (%) increase in the ischemic hemisphere vs. the contralateral hemisphere. Hb content was measured using brain homogenates prepared from TTC sections with Quanti-Chrom kit (BioAssay Systems, Hayward, CA), 2007) and reported as excess hemoglobin (Hb, µg/mg protein) in the ischemic hemisphere normalized to sham animals. Dead animals were counted for mortality rates.

Evaluation of MMP3 Expression

MMP3 expression in brain homogenates was analyzed by Western blot analysis. In brief, equal volumes of homogenized brain tissues (30 µg total protein) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. MMP3 was determined by using anti-MMP3 antibody (1:500)

(ab53015, Abcam, USA). Primary antibodies were detected using horseradish peroxidase-conjugated antibody and enhanced chemiluminescence. Band intensity was quantified by densitometry software (Alpha Innotech; Santa Clara, CA).

Evaluation of MMP3 Enzymatic Activity

The enzymatic activity of MMP-3 was determined using a fluorescence resonance energy transfer (FRET) peptide and immunocapture assay as previously described elsewhere with minor modifications [17]. Briefly, 50 µg total protein of macrovascular or brain homogenates were incubated at 4°C for 2 h with rabbit polyclonal anti-MMP-3 antibody (Cat.No. sc-6839-R; Santa Cruz Biotechnology, Dallas, TX, USA). A/G agarose beads were then added and allowed to incubate overnight at 4°C. The beads were then washed and samples transferred to black 96 well plate and 100 ul of 2mM 5-FAM/QXL™520 FRET peptide (Cat. No. 60580-01; AnaSpec, San Jose, CA, USA) in assay buffer were added per well. Plates were incubated for 8 h at 37°C, then relative fluorescence units (RFUs) were read and monitored at excitation/emission wavelengths of 485/528 nm in a Synergy HT Multi-mode microplate fluorescence reader (BioTek, Winooski, VT, USA) running Gen5™ data analysis software.

Immunohistochemistry and Co-localization Studies

At 24h, brains were isolated and coronal sections for immunohistochemistry were prepared following the standard methods [18]. Sections were incubated overnight with rabbit polyclonal anti-MMP-3 antibody (1:500, Cat.No. sc-6839-R; Santa Cruz Biotechnology,

Dallas, TX, USA) at 4°C overnight. For the co-localization study, brain sections were then incubated with either anti-NeuN antibody (ABN78, EMD Millipore, USA) as a neuronal marker , isolectin B4 (IB4, I21413, Invitrogen Life Technologies, USA) as endothelial markers, glial fibrillary acidic protein (GFAP) (Ab5804, Millipore, USA) as marker for astrocytes or NG2 (05-710, Millipore, USA) as a pericyte marker. This was followed by reaction with fluorescent conjugated secondary antibodies goat anti-rabbit (1:1000, Invitrogen Life Technologies, USA). Images were acquired from regions B, C and D (+3 to -3 mm from bregma) as regions of interest with maximal brain infarct area. Three slides were obtained from each brain with nine fields tested in the regions of interest within the cortex and striatum and the ischemic border zone. Brain sections were examined using Axiovert 200 Microscope (Carl Zeiss Micro-Imaging, Thornwood, NY, USA).

Statistical Analysis

Data are presented as mean ± SEM. A 2 HG (no vs. mild HG) by 6 Time (baseline, 15min preMCAO, 45min MCAO, 90min MCAO, 30min reperfusion, and 24h) repeated measures ANOVA was used to determine changes in blood glucose over time dependent on HG status. Stroke outcomes included MMP-3 activity in brain homogenate and macrovessels, percent infarct size and edema, HT index, excess Hb, and the functional outcomes composite score and grip strength. The effect of addition of MMP3 inhibitor was determined using a 1-way ANOVA with 3 groups (control, HG, HG+MMP3 inh). The effect of hyperglycemia and protein knock down by shRNA was assessed using a 2 HG (no vs. yes) by 2 shRNA (no vs. yes) ANOVA where a significant interaction would indicate a differential effect of shRNA on stroke outcomes dependent on HG status. SAS©

9.3 (SAS, Inc., Cary, NC) was used for all analyses. Statistical significance was determined at alpha<0.05 and a Tukey's post-hoc test was used to compare means from significant ANOVAs.

Results

Effect of HG on MMP3 Activity

The use of 30% glucose solution achieved and maintained blood glucose levels of 140-200 mg/dl through 90 minutes of ischemia, which is significantly higher than control normoglycemic animals (80-120 mg/dl) (Fig. 3.1A). Acute mild HG significantly increased MMP3 activity in cerebral macrovessels and brain homogenates and the use of MMP3 inhibitor (UK 356618) significantly reduced MMP3 activity in the brain (Fig. 3.1B and C).

Spatial Expression of MMP3 in NVU in Acute Hyperglycemic Stroke

MMP3 staining was more evident in the HG group and colocalized with NeuN positive neurons, isolectin positive cerebral vessels and NG2 positive pericytes in the peri-infarct region. However, MMP3 was not seen in GFAP positive astrocytes (Fig. 3.2).

The Effect of MMP3 Inhibition on Outcomes in Hyperglycemic Stroke

As previously reported, mild HG did not increase infarct size (Fig. 3.3A) but exacerbated vascular injury as indicated by greater edema and HT as compared to control group (Fig. 3.3B-D). The pharmacological inhibition of MMP3 significantly reduced HT, edema and

Hb content in ischemic hemispheres without a reduction in infarct size in hyperglycemic animals (Fig. 3.3A-D). This was associated with better neurobehavioral scores and improved grip strength (Fig. 3.4). The mortality rate was 10-15% and there was no significant difference in mortality between different groups.

The Effect of Knocking Down MMP3 in the Brain on Stroke Outcomes

Injection of MMP3 shRNA lentiviral particles significantly knocked down the expression of MMP3 in the brain compared to animals injected with the empty vector and sham animals (Fig. 3.5A). MMP3 knockdown significantly reduced brain edema without affecting the infarct size (Fig. 3.5 A and B). Furthermore, knocking down MMP3 significantly reduced HT and Hb content in the ischemic hemispheres in hyperglycemic animals and this was associated with significant increase in neurobehavioral composite scores and grip strength (Fig. 3.6).

Discussion

The goal of this study is to fill a gap in the knowledge about the mechanisms mediating HG-induced neurovascular injury and elucidate the role of MMP3 as one of the critical mediators of HT in hyperglycemic stroke. Accordingly, this study was designed to investigate: 1) the impact of HG on MMP3 activity and localization in the brain after stroke and 2) the impact of the HG induced elevation in MMP3 activity on HT and functional outcomes.

MMP3 is a zinc endopeptidase that has broad substrate specificity and can target mostly all the components of the neurovascular unit causing degradation of the basal lamina and tight junction proteins [9, 11]. Suzuki et al showed MMP3 is a critical mediator of the tPA induced cerebral hemorrhage which was significantly reduced in an MMP3 but not MMP9 knockout mouse model of normoglycemic animals [13]. We have shown in the current study that HG has significantly increased MMP3 activity in the brain after stroke. This HG-induced increase in MMP3 activity was associated with significant increase in cerebrovascular bleeding and HT. This can be related in part to the proteolytic ability of MMP3 to degrade TJ proteins leading to disruption of the blood brain barrier, swelling of the brain and cerebral hemorrhage.

Previous studies have shown that MMP3 is expressed in the neurons in the acute phase after stroke and the administration of tPA induced its expression in endothelial cells, however, it was not detected in the astrocytes. In our study, we detected MMP3 in the neurons after stroke, however, we didn't see an increase in the expression of MMP3 in the neurons with HG. On the other side, the expression of MMP3 in cerebral vessels was significantly upregulated with HG. MMP3 was more localized to the pericytes surrounding

the cerebral vessels and small portions of the vessels in normoglycemic animals. With HG, MMP3 expression was seen along the whole vessels body and surrounding pericytes. This spatial expression pattern of MMP3 in cerebral vessels and the surrounding pericytes in the peri-infarct region after stroke provides a support to our findings that MMP3 increase HT in hyperglycemic stroke. Our results showing that vascular injury is significantly reduced without a change in infarct size when MMP3 is inhibited further support vascular upregulation of MMP3 in our hyperglycemic stroke model.

We used two methods to block MMP3 activity. Acute pharmacological inhibition was achieved by single dose administration at reperfusion. Given that there was no information on the in vivo use of MMP3 inhibitor UK356618 in the literature, we first performed a series of dose-finding studies. We selected a dose that resulted in 50% or more reduction in MMP3 activity. Administration of the drug to sham operated animals did not reduce MMP3 activity suggesting that either the inhibitor does not cross blood brain barrier in the absence of ischemic injury or the MMP3 activity in sham animals was below the threshold of the drug action, as MMP3 showed very low activity in the brains of sham animals compared to control and HG stroked animals (supplemental data, Fig. 3.7). In order to address the concerns about the specificity of the inhibitor, we next used a molecular approach to knock down MMP3 expression by stereotaxic injection of MMP3 shRNA lentiviral particles directly into the lateral ventricles in the brain. We achieved approximately 50% reduction in MMP3 expression, similar to the decrease observed in MMP3 activity with the inhibitor. These results further support our finding that MMP3 is a critical mediator of vascular injury in hyperglycemic stroke.

Although this study can be considered the first to investigate the role of MMP3 in hyperglycemic stroke, the use of only male, young healthy animals and assessment of only short term outcomes remain to be limitations of this study. Moreover, we investigated only the effect of MMP3 without looking for other MMPs like MMP2 and MMP9. However, it was previously reported by our group that acute HG significantly increased MMP9 activity in the MCA in a similar model of stroke [7].

In conclusion, the significant improvement in the HT and functional outcomes achieved with the pharmacological inhibition and focal knockdown of MMP3 strongly support our hypothesis in demonstrating the deleterious role of MMP3 in mediating HT during the acute phase in hyperglycemic stroke. Taken together, this experimental mechanistic study has high translational impact in pointing out MMP3 as a potential therapeutic target for reducing cerebral bleeding and improving clinical outcomes in hyperglycemic stroke.

Source of Funding

Adviye Ergul is a Research Career Scientist at the Charlie Norwood Veterans Affairs Medical Center in Augusta, Georgia. This work was supported in part by VA Merit Award (BX000347), VA Research Career Scientists Award, and NIH (R01NS083559) to Adviye Ergul; VA Merit Award (BX000891) and NIH award (NS063965) to Susan C. Fagan, and American Heart Association Predoctoral Fellowship (13PRE17090026) to Sherif Hafez. The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

Disclosures

None.

Figure legends

Figure 3.1. Blood glucose and MMP3 activity. **A,** Administration of 30% glucose solution achieved and maintained blood glucose levels of 140-200 mg/dl through 90 minutes of ischemia. Acute mild HG significantly increased MMP3 activity in **B,** brain homogenates and **C,** cerebral vasculature after stroke and the use of MMP3 inhibitor significantly blunted this effect. *$p<0.05$ vs control, #$p<0.05$ vs HG, $^{\alpha}p<0.05$vs sham, n=7-9 per group.

Figure 3.1. Blood glucose and MMP3 activity.

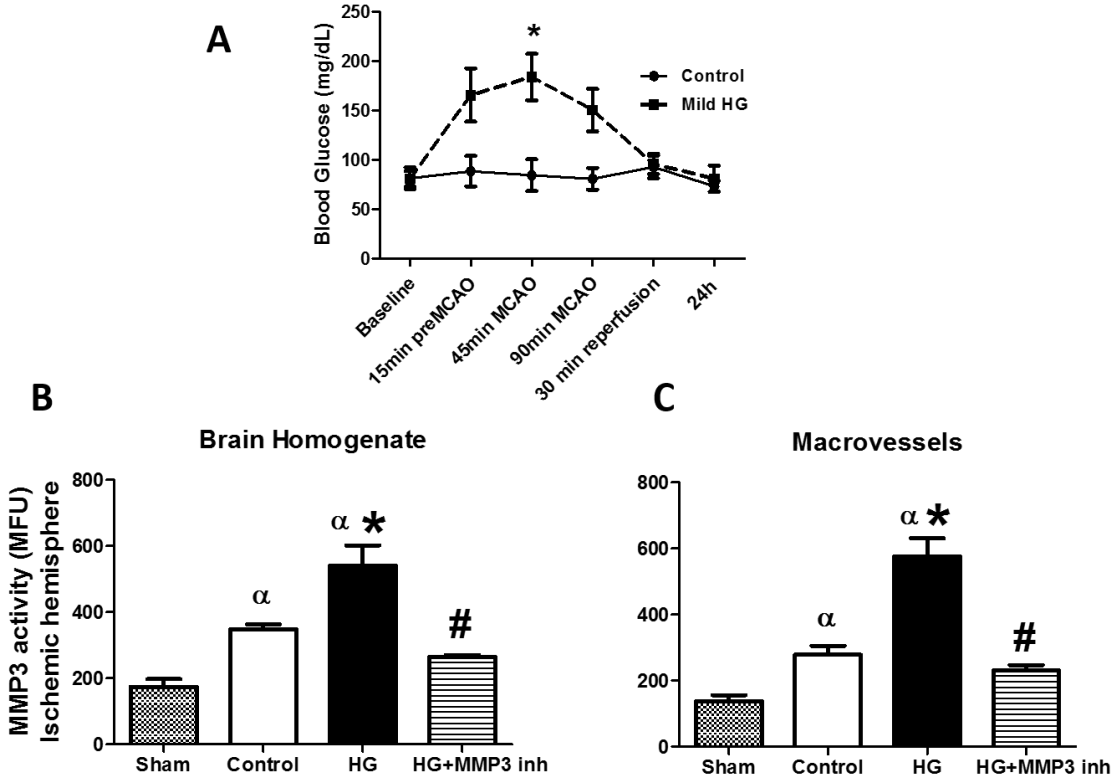
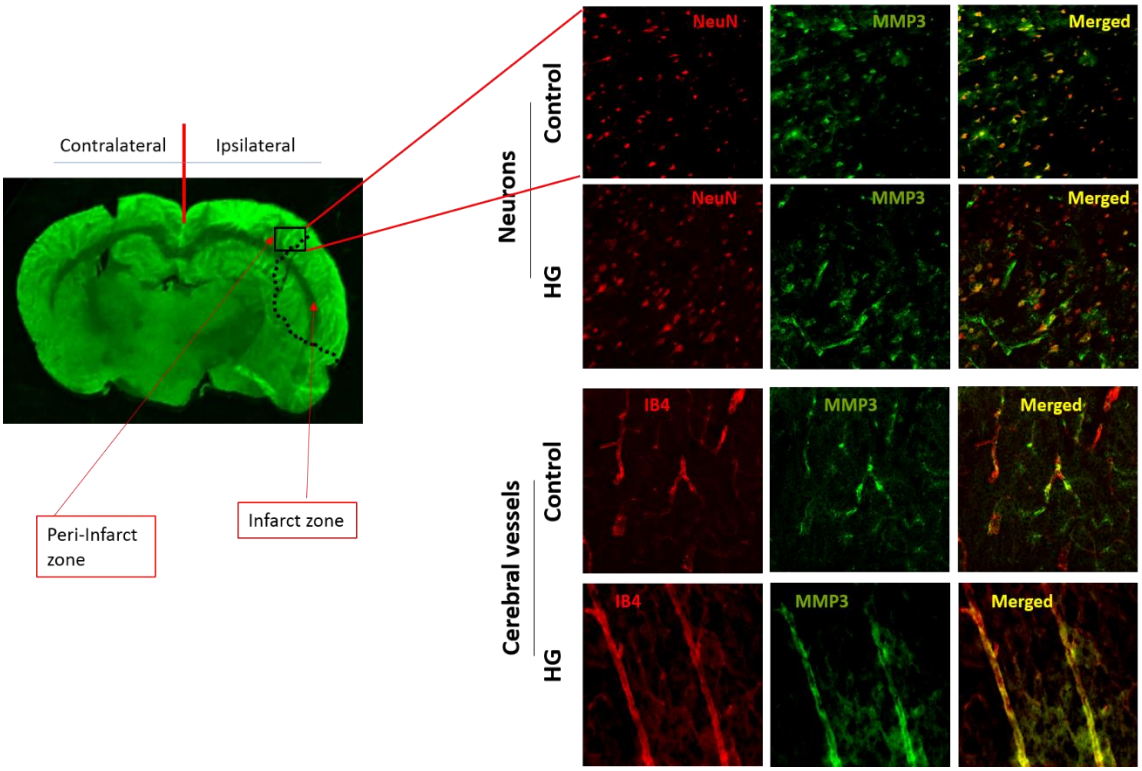


Figure 3.2. Localization of MMP3 in the neurovascular unit in hyperglycemic stroke at 24 h. **A,** MMP3 colocalizes with NeuN positive neurons and **B,** isolectin positive cerebral vessels and **C, NG2** positive pericytes in the peri-infarct region in both control and hyperglycemic groups. However, MMP3 was not seen in **D,** GFAP positive astrocytes.

Figure 3.2. Localization of MMP3 in the neurovascular unit in hyperglycemic stroke at 24 h.



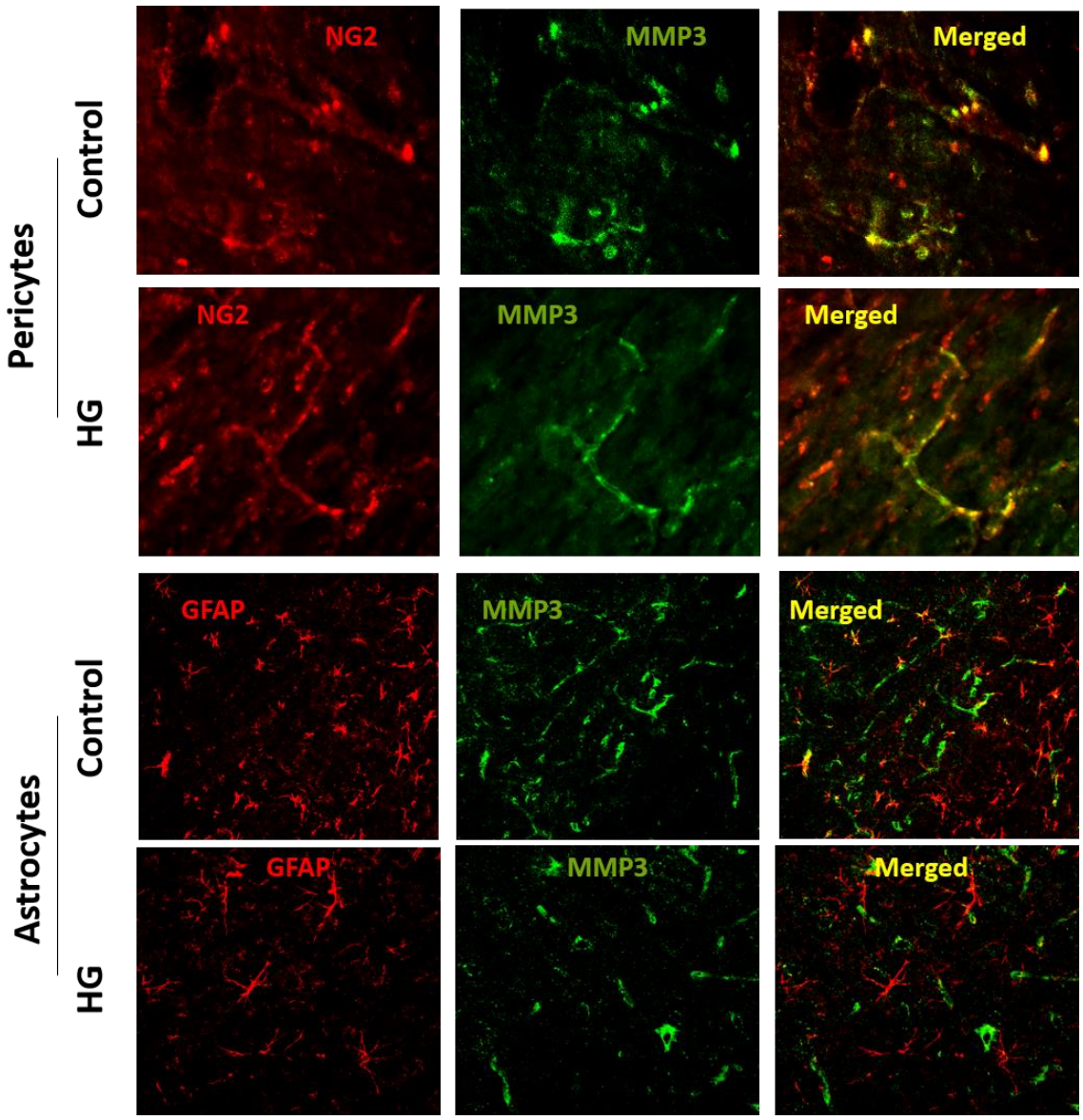

Pericytes
Control
HG
NG2
MMP3
Merged
NG2
MMP3
Merged
Astrocytes
Control
HG
GFAP
MMP3
Merged
GFAP
MMP3
Merged

Figure 3.3. MMP3 pharmacological inhibition and neurovascular injury. Acute HG significantly exacerbated the vascular injury and bleeding in the brain. MMP3 inhibition in hyperglycemic animals significantly reduced the HG induced **B,** brain edema, **C,** HT and **D,** Hb content in ischemic hemispheres without affecting the **A,** infarct size. *$p<0.05$ vs control, #$p<0.05$ vs HG, n=7-9 per group.

Figure 3.3. MMP3 pharmacological inhibition and neurovascular injury.

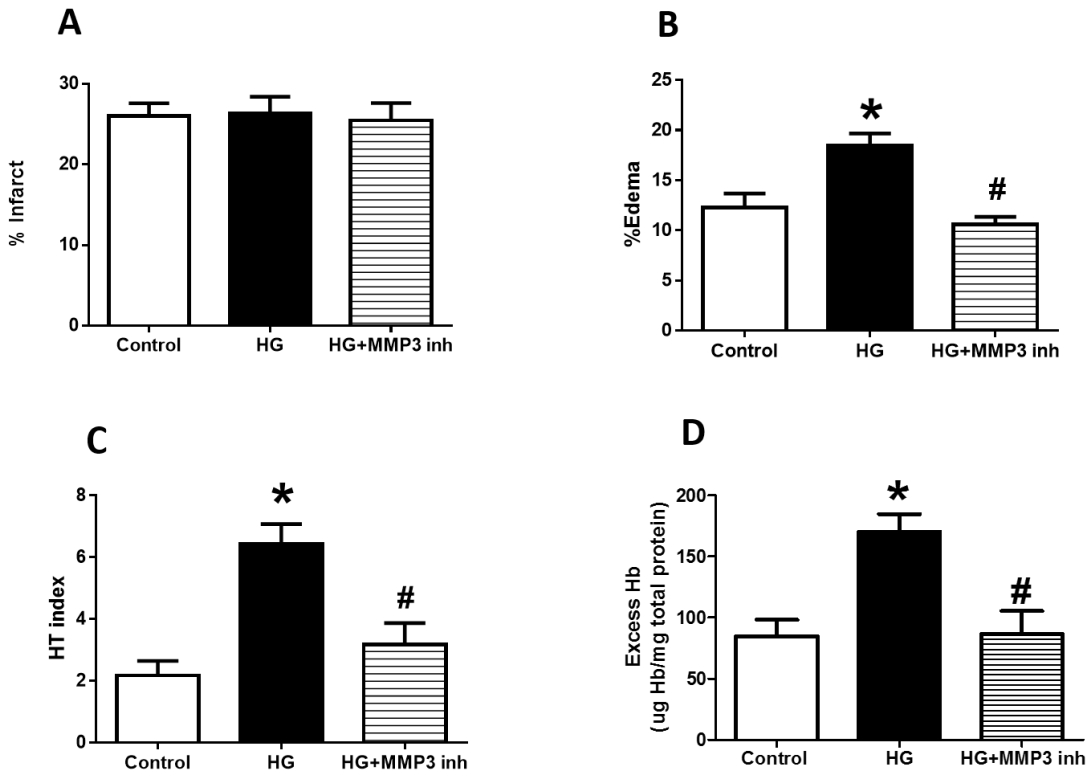


Figure 3.4. MMP3 pharmacological inhibition and functional outcomes. Acute mild HG significantly worsened the functional outcomes. Inhibition of MMP3 activity was associated with a significant increase in **A,** neurobehavioral composite score and **B,** grip strength in hyperglycemic animals. *$p<0.05$ vs control, #$p<0.05$ vs HG, $^{\alpha}p<0.05$vs pre-stroke, n=7-9 per group.

Figure 3.4. MMP3 pharmacological inhibition and functional outcomes.

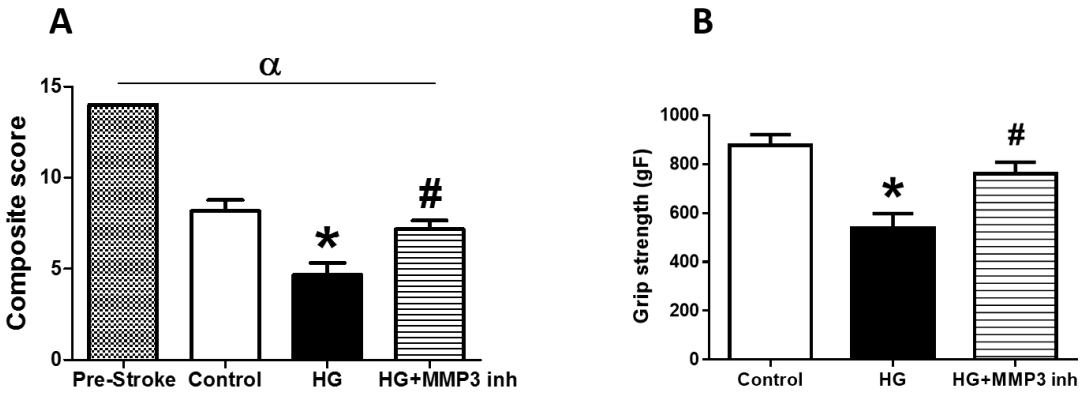


Figure 3.5. MMP3 knockdown in the brain. **A,** Stereotaxic injection of MMP3 shRNA lentiviral particles significantly knocked down the expression of MMP3 in the brain compared to sham or animals injected with empty vector ($^{\alpha}p<0.05$ vs sham and EV). MMP3 knockdown significantly reduced the HG induced **B,** brain swelling without affecting **A,** infarct size**.** *$p<0.05$ vs control EV and control shRNA, #$p<0.05$ vs HG EV, n=7-9 per group.

Figure 3.5. MMP3 knockdown in the brain.

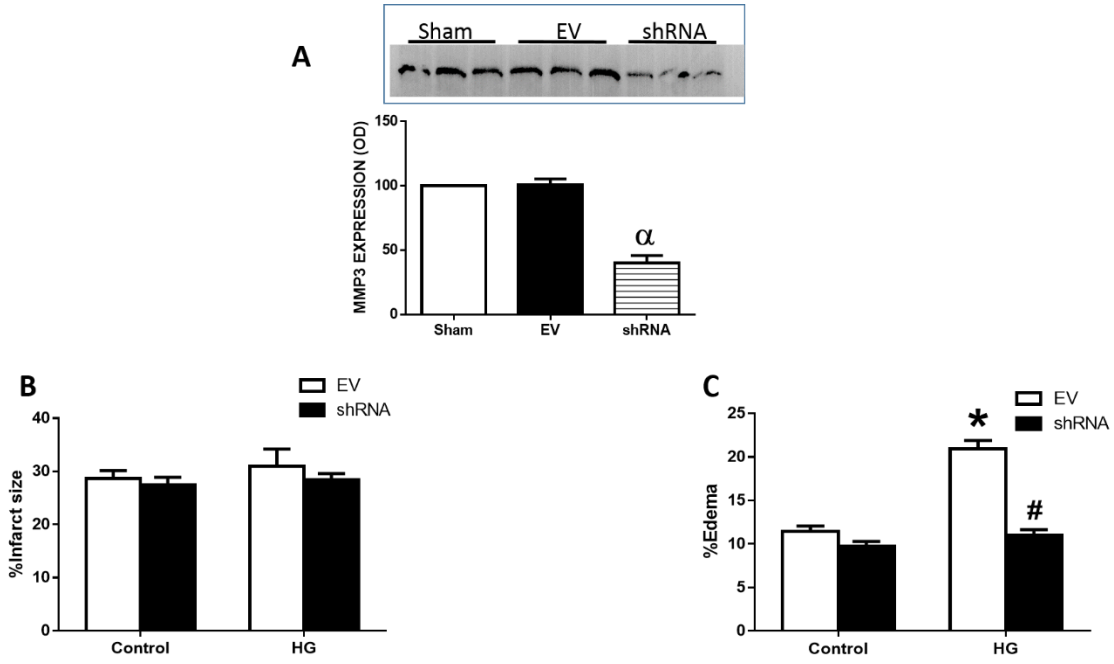


Figure 3.6. MMP3 focal knockdown in rat brain and stroke outcomes. Knocking down MMP3 in rats' brains significantly reduced the HG induced increase in **A,** HT and **B,** Hb content in ischemic hemispheres and improved the HG induced neurological deficits as indicated by a significant increase in **C,** neurobehavioral composite score and **D,** grip strength. *$p<0.05$ vs control EV and control shRNA, #$p<0.05$ vs HG EV, n=7-9 per group.

Figure 3.6. MMP3 focal knockdown in rat brain and stroke outcomes.

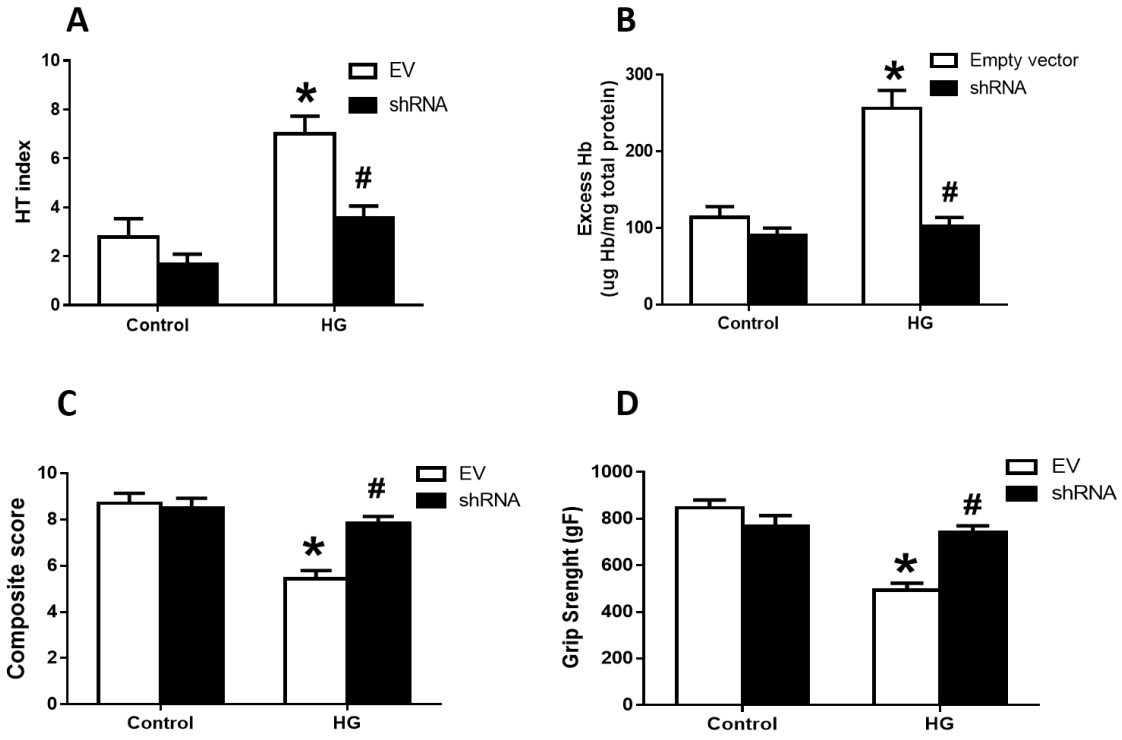
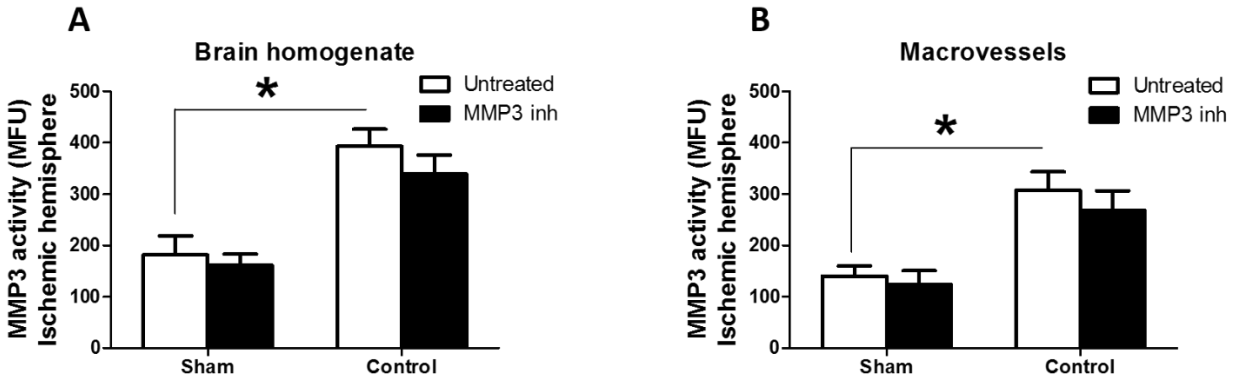


Figure 3.7. Supplemental figure 1. MMP3 activity in Sham and Control animals.

Control (stroked normoglycemic) animals showed significant increase in MMP3 activity in both **A,** brain homogenates and **B,** cerebral macrovessels compared to sham animals. The use of MMP3 inhibitor caused a slight yet insignificant reduction in MMP3 activity in control animals. However, no reduction in activity was seen in sham animals after treatment. *$p<0.05$ vs control untreated, n=4-6/gp.

Figure 3.7. MMP3 activity in Sham and Control animals.



BIBLIOGRAPHY

1. Roger, V.L., et al., *Heart disease and stroke statistics--2012 update: a report from the American Heart Association.* Circulation, 2012. **125**(1): p. e2-e220.
2. Bruno, A., et al., *Acute blood glucose level and outcome from ischemic stroke. Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators.* Neurology, 1999. **52**(2): p. 280-4.
3. Bruno, A., et al., *Admission glucose level and clinical outcomes in the NINDS rt-PA Stroke Trial.* Neurology, 2002. **59**(5): p. 669-74.
4. Capes, S.E., et al., *Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview.* Stroke, 2001. **32**(10): p. 2426-32.
5. Hafez, S., et al., *Hyperglycemia, Acute Ischemic Stroke, and Thrombolytic Therapy.* Transl Stroke Res, 2014.
6. Kruyt, N.D., et al., *Hyperglycemia in acute ischemic stroke: pathophysiology and clinical management.* Nat Rev Neurol, 2010. **6**(3): p. 145-55.
7. Elgebaly, M.M., et al., *Neurovascular injury in acute hyperglycemia and diabetes: A comparative analysis in experimental stroke.* Transl Stroke Res, 2011. **2**(3): p. 391-398.
8. Hafez, S., et al., *Comparative Analysis of Different Methods of Ischemia/Reperfusion in Hyperglycemic Stroke Outcomes: Interaction with tPA.* Transl Stroke Res, 2015.
9. Jin, R., G. Yang, and G. Li, *Molecular insights and therapeutic targets for blood-brain barrier disruption in ischemic stroke: critical role of matrix metalloproteinases and tissue-type plasminogen activator.* Neurobiol Dis, 2010. **38**(3): p. 376-85.
10. Lakhan, S.E., et al., *Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke.* Front Neurol, 2013. **4**: p. 32.
11. Kim, E.M. and O. Hwang, *Role of matrix metalloproteinase-3 in neurodegeneration.* J Neurochem, 2011. **116**(1): p. 22-32.
12. Sole, S., et al., *Activation of matrix metalloproteinase-3 and agrin cleavage in cerebral ischemia/reperfusion.* J Neuropathol Exp Neurol, 2004. **63**(4): p. 338-49.
13. Suzuki, Y., et al., *Stromelysin-1 (MMP-3) is critical for intracranial bleeding after t-PA treatment of stroke in mice.* J Thromb Haemost, 2007. **5**(8): p. 1732-9.
14. Fisher, M., et al., *Update of the stroke therapy academic industry roundtable preclinical recommendations.* Stroke, 2009. **40**(6): p. 2244-50.
15. Lapchak, P.A., J.H. Zhang, and L.J. Noble-Haeusslein, *RIGOR guidelines: escalating STAIR and STEPS for effective translational research.* Transl Stroke Res, 2013. **4**(3): p. 279-85.
16. Ergul, A., et al., *Increased hemorrhagic transformation and altered infarct size and localization after experimental stroke in a rat model type 2 diabetes.* BMC Neurol, 2007. **7**: p. 33.
17. Candelario-Jalil, E., et al., *Matrix metalloproteinases are associated with increased blood-brain barrier opening in vascular cognitive impairment.* Stroke, 2011. **42**(5): p. 1345-50.
18. Zhao, B.Q., et al., *Role of matrix metalloproteinases in delayed cortical responses after stroke.* Nat Med, 2006. **12**(4): p. 441-5.

CHAPTER 4

HYPERGLYCEMIA MEDIATES MATRIX METALLOPROTEASE 3 (MMP3) ACTIVATION THROUGH PEROXYNITRITE INDUCED TYROSINE NITRATION AFTER STROKE [3]

[3] Sherif Hafez, Mohammed Abdelsaid, Susan C. Fagan and Adviye Ergul.

To be submitted.

HYPERGLYCEMIA MEDIATES MATRIX METALLOPROTEASE 3 (MMP3) ACTIVATION THROUGH PEROXYNITRITE INDUCED TYROSINE NITRATION AFTER STROKE

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Abstract

Background and Purpose. Tissue plasminogen activator (tPA) was shown to induce hemorrhagic transformation (HT) after stroke and the incidence of hyperglycemia further exacerbates this outcome. The interaction between tPA and HG in activating matrix metalloproteinase 3 (MMP3) after stroke was not previously reported. Accordingly, in this study we are investigating the impact of and the mechanisms through which tPA and HG activate MMP3.

Methods. Control/normoglycemic and hyperglycemic (blood glucose: 140-200 mg/dl) male Wistar rats were subjected to middle cerebral artery occlusion (MCA) suture occlusion for 90 minutes or thromboembolic occlusion and up to 24 h reperfusion with and without tPA. MMP3 activity and MMP3 tyrosine nitration were evaluated in brain homogenates at 24 h. Brain vascular endothelial cells (BVEC) were subjected to 3 h hypoxia under either normoxic or hypoxic conditions with or without tPA and MMP3 activity and MMP3 tyrosine nitration were assessed at 24 h.

Results. HG and tPA significantly increased MMP3 activity in the brain after stroke and in BVECs after hypoxia/reoxygenation. HG significantly increased MMP3 tyrosine nitration in rats subjected to either suture or thromboembolic occlusion as well as BVECs.

Conclusion. HG and tPA significantly increased MMP3 activity in the brain after stroke and this was associated with increased MMP3 nitration. Based on previous literature, augmented oxidative and nitrative stress may be the underlying mechanisms of MMP3 activation in hyperglycemic stroke, especially with tPA administration. Correlating this to our previous findings that the combination of tPA and HG exacerbates HT and worsens the

functional outcomes, we point out MMP3 as a critical mediator of the tPA and HG induced neurovascular injury after stroke.

Introduction

Tissue plasminogen activator (tPA) is the only FDA approved thrombolytic therapy for acute ischemic stroke (AIS) patients. However, its use in the clinical setting is limited due to its narrow therapeutic window and its ability to increase the risk of intracranial hemorrhage (ICH) [1]. Acute Hyperglycemia (HG) was shown to be an independent predictor of worse clinical outcomes after stroke. Almost 25% of AIS patients are presented to the hospital with acute elevation in blood glucose levels and it was shown that those patients suffer the worst clinical outcomes when compared to normoglycemic or even diabetic patients [2-4]. Moreover, HG was shown to exacerbate the tPA induced hemorrhagic transformation (HT) [5, 6].

A large body of evidence from preclinical experimental studies showed the detrimental effects of HG on stroke outcomes and this was mainly attributed to the HG induced elevation in oxidative stress and inflammatory cytokines [7-9].

MMP3 has been shown to play a critical role in mediating the tPA induced intracerebral hemorrhage after stroke [10]. We have recently shown that MMP3 activity is increased in hyperglycemic stroke and its inhibition was associated with reduced vascular injury and improved functional outcomes. However, the impact of the interaction between HG and tPA on MMP3 activation remained unknown.

Peroxynitrite is a potent oxidizing and nitrating agent that can cause lipid peroxidation and protein nitration and consequently can be involved in BBB disruption. Peroxynitrite is upregulated in the brain after the ischemic injury and HG augments this effect [11, 12]. It was recently shown that peroxynitrite plays a role in MMP3 activation through tyrosine nitration in the synovial fluid of patient with temporomadibular joint (TMJ) disorders [13].

However, the mechanism through which MMP3 is activated in hyperglycemic stroke was not previously studied. Accordingly, this study was designed to test the hypothesis that: 1) tPA and HG interaction increases MMP3 activity after stroke; 2) peroxynitrite upregulates MMP3 activity through tyrosine nitration in hyperglycemic stroke.

Materials and Methods

Animal Models and Study Groups

The animals were housed at the Georgia Regents University animal care facility, which is approved by the American Association for Accreditation of Laboratory Animal Care. This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research and all protocols were approved by the institutional animal care and use committee.

Study 1. Determine the effect of HG and tPA on MMP3 activity.

Study 1.1 Determine the effect of HG and tPA on MMP3 activity *in vivo* in hyperglycemic stroke.

Control/Normoglycemic (NG) or mild HG (140 – 200 mg/dl) male Wistar rats (320 g) were subjected to either 90 minutes suture occlusion or thromboembolic occlusion of the MCA and up to 24 h reperfusion. Animals were randomized to receive either tPA (Cathflo Activase (Alteplase), Genentech) or vehicle (water for injection) of equal volume. tPA (1mg/Kg) was intravenously infused over 20 minutes through the jugular vein 2h after induction of ischemia to rats with either suture or thromboembolic occlusion. At 24h,

animals were sacrificed, brains were isolated and homogenized and examined for the MMP3 activity.

Study 1.2 Determine the effect of HG and tPA on MMP3 activity in brain vascular endothelial cells (BVEC).

BMVCs were cultured in DMEM and incubated in hypoxic chamber under low oxygen tension for 3 hours and then further cultured in DMEM with normal glucose (NG = 5 mM) or high glucose (HG = 25 mM) for 22.5 hours to mimic the in vivo model. Study groups are: 1) Normal glucose (NG); 2) NG + tPA; 3) High glucose (HG); 4) HG + tPA. Control cells were cultured in DMEM with normal or high glucose for 24 hours under normoxia. tPA was added (10 µg/ml) at the end of 3 h OGD or normoxia. At the end of the incubation period (24 h), supernatant and cell lysates were collected separately and MMP3 activity was measured.

Study 2. Determine the role of peroxynitrite in activating MMP3 through tyrosine nitration in hyperglycemic stroke.

Study 2.1. In vivo. Brain homogenates from study 1.1 were analyzed for MMP3 nitration by immunoprecipitation.

Study 2.2. In vitro. BVECs homogenates from study 1.2 were analyzed for MMP3 nitration.

Stroke Surgery

Focal cerebral ischemia was performed as previously described [14]. Briefly, A midline cervical incision was made to expose the common carotid artery. The external carotid artery (ECA) was separated, ligated and cauterized. An arteriotomy was performed on the ECA stump. A rounded-tip 3-0 monofilament nylon suture (prepared carefully under microscope with high magnification power to ensure uniformity) was inserted into the ECA stump and advanced through the internal carotid artery to occlude the origin of MCA. For the embolic model, the surgical procedure remained the same as in suture model except that instead of the monofilament suture, a PE-10 catheter containing the clot was inserted through the ECA stump to deliver the clot to the origin of the MCA. Scanning laser Doppler (Pim-3, Perimed, ST) was used to confirm a similar degree of drop in CBF among groups. The percent drop in CBF after stroke was determined by comparing to baseline [15]. Animals with reduction in CBF less than 40% from baseline are excluded.

Preparation of Blood Clots

Fresh blood clots were prepared as we previously reported in details [16-18].

Evaluation of MMP3 Enzymatic Activity

The enzymatic activity of MMP-3 was determined using a fluorescence resonance energy transfer (FRET) peptide and immunocapture assay as previously described elsewhere with minor modifications [19]. Briefly, 50 µg total protein of homogenized brain tissue were

incubated at 4°C for 2 h with rabbit polyclonal anti-MMP-3 antibody (Cat.No. sc-6839-R; Santa Cruz Biotechnology, Dallas, TX, USA). A/G agarose beads were then added and allowed to incubate overnight at 4°C. The beads were then washed and samples are transferred to black 96 well plate and 100 ul of 2mM 5-FAM/QXL™520 FRET peptide (Cat. No. 60580-01; AnaSpec, San Jose, CA, USA) in assay buffer were added per well. Plates were incubated for 8 h at 37°C, then relative fluorescence units (RFUs) were read and monitored at excitation/emission wavelengths of 485/528 nm in a Synergy HT Multi-mode microplate fluorescence reader (BioTek, Winooski, VT, USA) running Gen5™ data analysis software.

Immuno-precipitation and Western Blotting

MMP3 nitration was evaluated by immuno-precipitation (IP) of either rats' brain homogenates or BVECs cell lysates for MMP3 using anti-MMP3 antibody followed by western blotting against nitrated protein using anti-nitrotyrosine antibody. Briefly, 100 ug protein from either brain homogenates or BVECs lysates were incubated with anti-MMP3 antibody (Cat.No. sc-6839-R; Santa Cruz Biotechnology, Dallas, TX, USA) for 4 h at 4°C. A/G agarose beads were then added and allowed to incubate overnight at 4°C. On the next day, the Anti-MMP3-A/G protein complex was washed 2 times then immune-precipitated by centrifugation and then analyzed by Western blotting using anti-nitrotyrosine antibody. In brief, 50 ul of 2X loading buffer was added to the immune-precipitated protein-beads complex and boiled for 20 minutes then centrifuged and equal volumes of the supernatant (30 ul) were loaded and separated by by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. MMP3 nitration was

determined by using anti-nitrotyrosine antibody (1:500) (05-233, Millipore, USA). Primary antibodies were detected using horseradish peroxidase-conjugated antibody and enhanced chemiluminescence. Band intensity was quantified by densitometry software (Alpha Innotech; Santa Clara, CA). Western blotting for MMP3 in BVEC was done the same way using anti MMP3 antibody (1:500) (ab53015, Abcam, USA).

Statistical Analysis

The effect of hyperglycemia and tPA treatment on MMP3 activity was assessed using a 2 HG (no vs. yes) by 2 tPA (no vs. yes) ANOVA where a significant interaction would indicate a differential effect of tPA on MMP3 activity dependent on HG status. The effect of tPA on MMP3 activity in BVEC compared to untreated cells was determined using a student unpaired t-test. The effect of HG on MMP3 tyrosin nitration (OD) compared to NG groups was determined using a student unpaired t-test. The effect of HG and hypoxia treatment on MMP3 tyrosine nitration in BVEC was assessed using a 2 HG (no vs. yes) by 2 hypoxia (no vs. yes) ANOVA where a significant interaction would indicate a differential effect of hypoxia on MMP3 activity dependent on HG status. Statistical significance was determined at alpha<0.05 and a Tukey's post-hoc test was used to compare means from significant ANOVAs.

Results

The effect of HG and tPA on MMP3 activity

Compared to normoglycemic animals, HG significantly increased MMP3 activity in the brain. The administration of tPA significantly increased MMP3 activity compared to non-treated animals. The combination of HG and tPA significantly increased MMP3 activity more than each alone in rats subjected to either suture or thromboembolic occlusion (Figure 4.1). In vitro, the administration of tPA significantly increased MMP3 activity in BVEC under normal or high glucose conditions whether these cells were subjected to either normoxia or hypoxia. This effect was detected in both BVECs lysate and supernatant (Figure 4.2).

The effect of HG on MMP3 nitration in vivo

HG significantly increased MMP3 tyrosine nitration in the brain after stroke in rats subjected to either suture or thromboembolic occlusion (Figure 4.3).

The effect of HG and hypoxia on MMP3 expression and nitration in vitro

In BVECs, either high glucose or hypoxia alone significantly increased MMP3 expression (Figure 4.4A) and MMP3 nitration (Figure 4.4B) compared to cells subjected to normoxia normal glucose. The combination of hypoxia and HG significantly increased the MMP3 expression and nitration more than each alone (Figure 4.4A,B).

Discussion

Hyperglycemia is considered to be an independent predictor of poor clinical outcomes after stroke in both clinical trials and experimental preclinical studies. Moreover, it was shown also on both levels that HG exacerbates the tPA induced cerebral hemorrhage after stroke [5, 20, 21].

MMP3 is a zinc endopeptidase that has a broad substrate specificity and can target all the component of the neurovascular unit and cause disruption of the BBB [22]. It was previously shown that MMP3 plays a critical role in mediating the tPA induced ICH in mice after stroke [10]. However, the role of tPA and HG in activating MMP3 as a mediator of HT after stroke and the underlying mechanism through which HG increases the activity of MMP3 were not previously studied. Accordingly this study was designed to 1- Study the role of HG and tPA in activating MMP3 after stroke. 2- Study the role of peroxynitrite in activating MMP3 through tyrosine nitration in hyperglycemic stroke.

We have recently shown that either HG or tPA exacerbates the vascular injury, HT and consequently worsens the functional outcomes in a clinically relevant rat model of stroke. Moreover, the combination of HG and tPA further exacerbated the injury more than each alone in rats subjected to either suture or thromboembolic occlusion [18]. In the current study we are showing that either HG or tPA alone has increased the MMP3 activity in the brains of rats subjected to either suture or embolic occlusion and the combination of HG and tPA significantly increased the MMP3 activity more than each alone. Correlating this to our previous findings, we can conclude that MMP3 may be playing an important role in mediating the HG-tPA induced HT and worse functional outcomes after stroke.

Our findings also suggested that administration of tPA increases the activity of MMP3 in BVECs isolated from rat brain, whether these cells were cultured in either normal or high glucose conditions, subjected to either normoxia or hypoxia. In contrast to our hypothesis, there was no further increase in MMP3 activity under hypoxic high glucose conditions. This may be because the insult was not strong enough to trigger this effect, as we subjected the cells to only 3 hours of hypoxia. In 2009, Suzuki et al showed that administration of tPA has increased the production of MMP3 in brain endothelial cells derived from mouse brain subjected to 6 hours of glucose oxygen deprivation (OGD). However, this effect was not seen when the cells were subjected to only 3 h of OGD [23].

The underlying mechanisms through which HG worsens the neurovascular injury after stroke were always attributed to the HG induced production of reactive oxygen/nitrogen species especially superoxide anions and peroxynitrite.

Given that HG and stroke increase oxidative stress, we hypothesized that peroxynitite induced tyrosine nitration of MMP3 may be contributing to greater MMP3 activity in hyperglycemic stroke. In 2009, Fujita et al showed that MMP3 activation through tyrosine nitration takes place in the synovial fluid of patients with TMJ disorders and thus leading to the pathophysiological progression of the internal derangement (ID) of TMJ [13]. Our immuno-precipitation studies showed that HG increased MMP3 nitration in the brains of rats subjected to either suture or thromboembolic occlusion. This increase in MMP3 tyrosine nitration corresponded to an increase in MMP3 activity. However, this was not the case in BVECs, as cells subjected to hypoxia combined with high glucose showed an increase in MMP3 expression and MMP3 tyrosine nitration without a corresponding increase in activity. These findings show that tyrosine nitration may be one but not the only

underlying mechanism causing MMP3 activation in hyperglycemic stroke. However, still further studies are needed to investigate the impact of tPA administration on MMP3 nitration and to determine whether inhibition of nitration would prevent the increase in MMP3 activity.

Conclusion and Limitations

The mediators of HT were always vague and this constituted a big gap in the field of hyperglycemic stroke. And although this study is the first to investigate the role of tPA and HG induced activation of MMP3 in hyperglycemic stroke, there are still some limitations to be addressed. The use of only male, young healthy animals and studying only short term outcomes remain to be limitations of this study. Moreover, we didn't address the impact of tPA on MMP3 tyrosine nitration correlated to its increase in activity.

In conclusion, we have shown in the current study that the combination of HG and tPA significantly increased MMP3 activity after stroke and its activity was increased through peorxynitrite induced tyrosine nitration. Previously we have shown that the combination of HG and tPA has significantly exacerbated the HT and worsened the functional outcomes after stroke in different models of ischemia/reperfusion [18]. So, correlating the current findings to our previous ones, we conclude that MMP3 can be a critical mediator of the tPA and HG induced HT after stroke. Accordingly, we point out MMP3 as a potential therapeutic target in hyperglycemic stroke

Figure Legends

Figure 4.1. The effect of HG and tPA on MMP3 activity in the brain after stroke.

HG significantly increased MMP3 activity in the brain. The administration of tPA significantly increased MMP3 activity compared to non- treated animals. The combination of HG and tPA significantly increased MMP3 activity more than each alone in rats subjected to either suture or thromboembolic occlusion. *$p<0.05$ vs control vehicle, #$p<0.05$ vs HG vehicle and control tPA, n=6/gp.

Figure 4.1. The effect of HG and tPA on MMP3 activity in the brain after stroke.

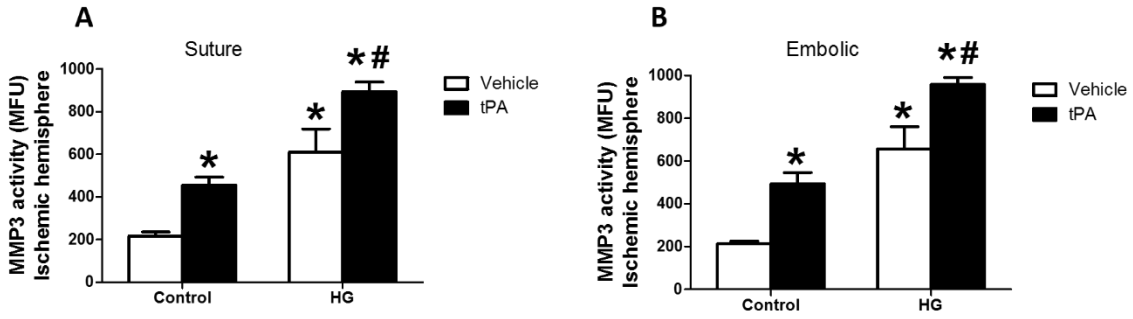


Figure 4.2. The effect of HG and tPA on MMP3 activity in BVEC after Hypoxia/reoxygenation.

tPA significantly increased MMP3 activity in BVEC under normal or high glucose conditions whether cells were subjected to either normoxia or hypoxia. This effect was detected in both BVECs lysate and supernatant. *$p<0.05$ vs non-tPA, n=4/gp.

Figure 4.2. The effect of HG and tPA on MMP3 activity in BVEC after hypoxia/reoxygenation.

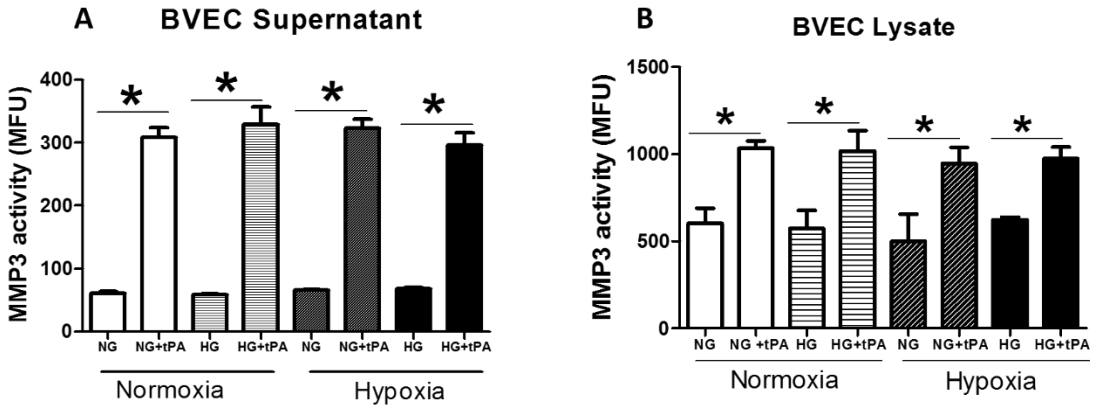


Figure 4.3. The effect of HG on MMP3 nitration in the brain after stroke.

HG significantly increased MMP3 tyrosine nitration in the brain after stroke in rats subjected to either suture or thromboembolic occlusion. *$p<0.05$ vs NG, n=6/gp.

Figure 4.3. The effect of HG on MMP3 nitration in the brain after stroke.

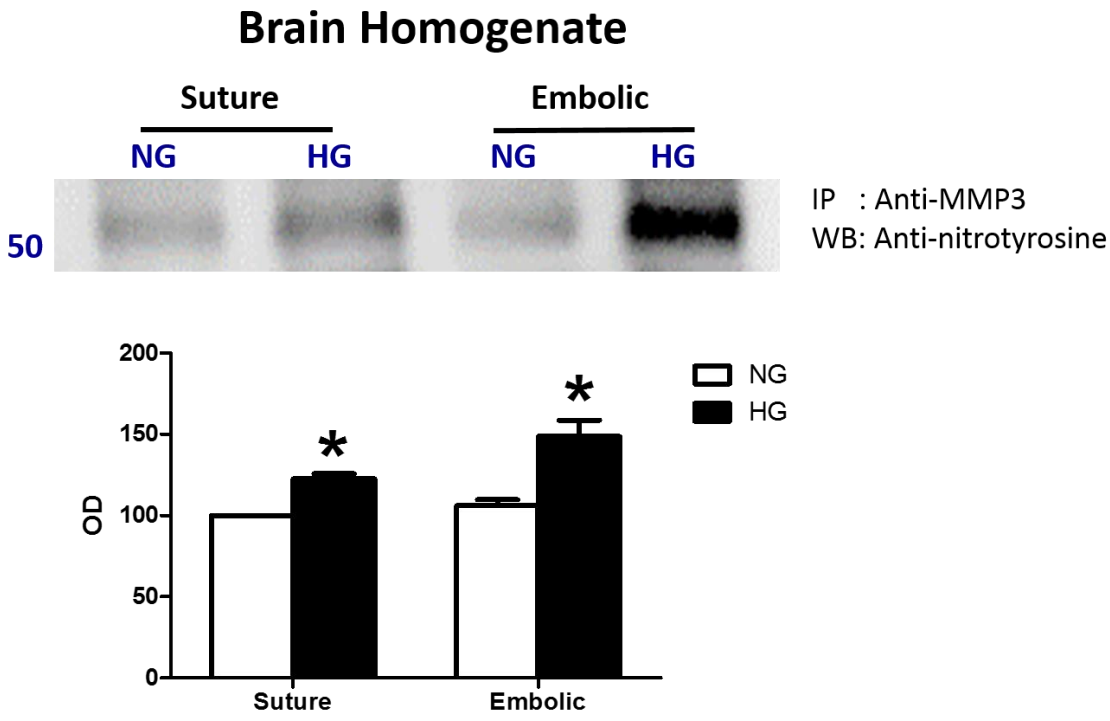
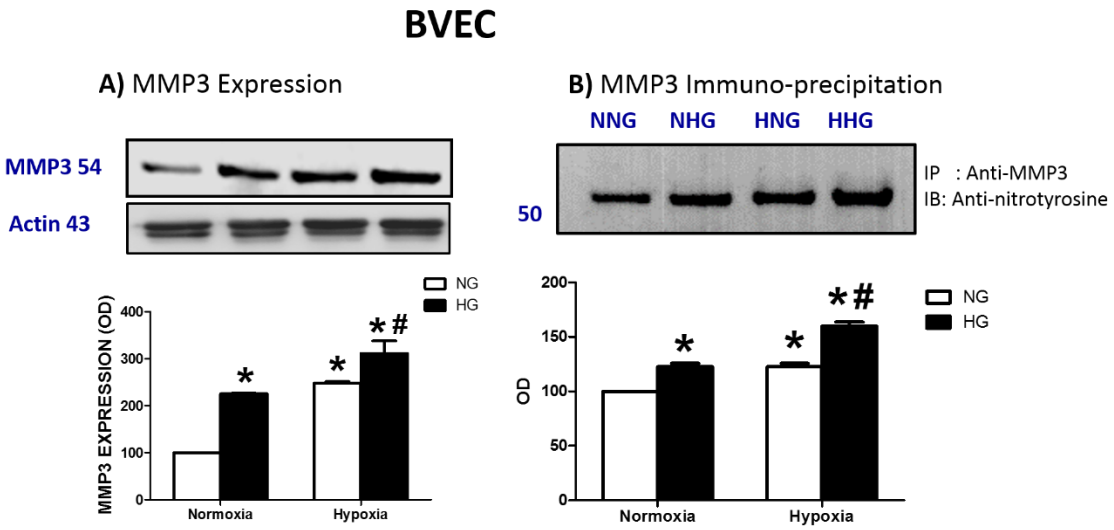


Figure 4.4. The effect of HG on MMP3 expression and MMP3 nitration in BVECs after hypoxia/reoxygenation.

High glucose and hypoxia alone significantly increased MMP3 expression and MMP3 nitration in BVECs compared to cells subjected to normoxia normal glucose. The combination of hypoxia and HG significantly increased the MMP3 expression and MMP3 nitration more than each alone. *$p<0.05$ vs normoxia NG, #$p<0.05$ vs hypoxia NG, normoxia HG. N=4/gp.

Figure 4.4. The effect of HG on MMP3 expression and MMP3 nitration in BVECs after hypoxia/reoxygenation.



BIBLIOGRAPHY

1. Fugate, J.E. and A.A. Rabinstein, *Update on intravenous recombinant tissue plasminogen activator for acute ischemic stroke.* Mayo Clin Proc, 2014. **89**(7): p. 960-72.
2. Bruno, A., et al., *Acute blood glucose level and outcome from ischemic stroke. Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators.* Neurology, 1999. **52**(2): p. 280-4.
3. Bruno, A., et al., *Admission glucose level and clinical outcomes in the NINDS rt-PA Stroke Trial.* Neurology, 2002. **59**(5): p. 669-74.
4. Capes, S.E., et al., *Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: a systematic overview.* Stroke, 2001. **32**(10): p. 2426-32.
5. Alvarez-Sabin, J., et al., *Effects of admission hyperglycemia on stroke outcome in reperfused tissue plasminogen activator--treated patients.* Stroke, 2003. **34**(5): p. 1235-41.
6. Poppe, A.Y., et al., *Admission hyperglycemia predicts a worse outcome in stroke patients treated with intravenous thrombolysis.* Diabetes Care, 2009. **32**(4): p. 617-22.
7. Hafez, S., et al., *Hyperglycemia, Acute Ischemic Stroke, and Thrombolytic Therapy.* Transl Stroke Res, 2014.
8. Kamada, H., et al., *Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction.* Stroke, 2007. **38**(3): p. 1044-9.
9. Kruyt, N.D., et al., *Hyperglycemia in acute ischemic stroke: pathophysiology and clinical management.* Nat Rev Neurol, 2010. **6**(3): p. 145-55.
10. Suzuki, Y., et al., *Stromelysin-1 (MMP-3) is critical for intracranial bleeding after t-PA treatment of stroke in mice.* J Thromb Haemost, 2007. **5**(8): p. 1732-9.
11. Bemeur, C., L. Ste-Marie, and J. Montgomery, *Increased oxidative stress during hyperglycemic cerebral ischemia.* Neurochem Int, 2007. **50**(7-8): p. 890-904.
12. Lehner, C., et al., *Oxidative stress and blood-brain barrier dysfunction under particular consideration of matrix metalloproteinases.* Antioxid Redox Signal, 2011. **15**(5): p. 1305-23.
13. Fujita, H., et al., *MMP-3 activation is a hallmark indicating an early change in TMJ disorders, and is related to nitration.* Int J Oral Maxillofac Surg, 2009. **38**(1): p. 70-8.
14. Ergul, A., et al., *Increased hemorrhagic transformation and altered infarct size and localization after experimental stroke in a rat model type 2 diabetes.* BMC Neurol, 2007. **7**: p. 33.
15. Guan, W., et al., *Acute Treatment with Candesartan Reduces Early Injury After Permanent Middle Cerebral Artery Occlusion.* Translational Stroke Research, 2011: p. 1-7.
16. Hoda, M.N., et al., *Sex-independent neuroprotection with minocycline after experimental thromboembolic stroke.* Exp Transl Stroke Med, 2011. **3**(1): p. 16.
17. Li, W., et al., *Comparative analysis of the neurovascular injury and functional outcomes in experimental stroke models in diabetic Goto-Kakizaki rats.* Brain Res, 2013. **1541**: p. 106-14.

18. Hafez, S., et al., *Comparative Analysis of Different Methods of Ischemia/Reperfusion in Hyperglycemic Stroke Outcomes: Interaction with tPA.* Transl Stroke Res, 2015.
19. Candelario-Jalil, E., et al., *Matrix metalloproteinases are associated with increased blood-brain barrier opening in vascular cognitive impairment.* Stroke, 2011. **42**(5): p. 1345-50.
20. Fan, X., et al., *A rat model of studying tissue-type plasminogen activator thrombolysis in ischemic stroke with diabetes.* Stroke, 2012. **43**(2): p. 567-70.
21. Won, S.J., et al., *Hyperglycemia promotes tissue plasminogen activator-induced hemorrhage by Increasing superoxide production.* Ann Neurol, 2011. **70**(4): p. 583-90.
22. Kim, E.M. and O. Hwang, *Role of matrix metalloproteinase-3 in neurodegeneration.* J Neurochem, 2011. **116**(1): p. 22-32.
23. Suzuki, Y., et al., *Tissue-type plasminogen activator (t-PA) induces stromelysin-1 (MMP-3) in endothelial cells through activation of lipoprotein receptor-related protein.* Blood, 2009. **114**(15): p. 3352-8.

CHAPTER 5

INTEGRATED DISCUSSION

Admission HG is a major finding in acute ischemic stroke patients and it leads to worse clinical outcomes [1]. In most if not all the conducted clinical trials, HG was shown to be an independent predictor of poor neurological outcomes, increased incidence of symptomatic intracranial hemorrhage and death [1-3]. Moreover, HG was shown to exacerbate the tPA induced HT after stroke [4, 5]. However, the underlying mechanisms through which HG induces HT after stroke are not fully understood and this constitutes a significant deficit in the hyperglycemic stroke field.

The goal of this dissertation is to investigate the underlying mechanisms through which HG and tPA mediates HT after stroke and to point out MMP3 as a novel therapeutic target in hyperglycemic stroke through establishing a mechanistic link between HG, MMP3 and HT. Accordingly, we studied: 1- The impact of the severity of acute HG on stroke outcomes using clinically relevant experimental stroke models and blood glucose levels; 2- The impact of the interaction between acute HG and tPA on neurovascular injury and functional outcomes in different experimental stroke models that mimic the clinical scenario; 3- The role of MMP3 in mediating the HG and tPA induced HT; and 4- The role of the HG induced peroxynitrite in activating MMP3 through tyrosine nitration after stroke.

First, we studied the impact of acute HG on stroke outcomes and our findings provided evidence that the severity of HG is a determinant of the neurovascular injury and functional outcomes after stroke. We found that mild HG (140-200 mg/dl) increased edema, HT and bleeding into the brain without increasing the infarct size and this was associated with

worse functional outcomes. This reflects the sensitivity and the vulnerability of the vasculature to acute yet subtle changes in blood glucose and also reflects the role the vasculature plays in contributing to worse functional outcomes when there is no further neuronal injury. However, severe HG (240-350 mg/dl) significantly increased the infarct size compared to normoglycemic and even mildly hyperglycemic animals and this was associated with further decline in the neurobehavioral outcomes. The major cause of this increase in the infarct size may be the massive increase in the HG induced production of reactive oxygen species and inflammatory cytokines. The previous preclinical studies employed severely high and clinically irrelevant blood glucose levels (350-500 mg/dl) to evaluate the impact of HG on stroke outcomes. However, findings from the THIS (Treatment of hyperglycemia in Ischemic Stroke) and GRASP (Glucose Regulation in Acute Ischemic Stroke Patients) clinical trials which were conducted to evaluate the management of HG in ischemic stroke demonstrated that admission blood glucose levels of AIS patients range between 160-240 mg/dl [6, 7]. Accordingly we designed our studies to employ clinically relevant blood glucose levels.

We found from the first studies that mild elevation in blood glucose was sufficient to exacerbate the vascular injury and worsen the functional outcomes. Accordingly, we employed these blood glucose levels for the next set of studies to evaluate the impact of the interaction between HG and tPA and the method of reperfusion on stroke outcomes. We found that either HG or tPA alone significantly exacerbated the HT, bleeding and edema into brain without affecting the infarct size in rats subjected to either suture or thromboembolic occlusion. The combination of HG and tPA further exacerbated the injury and worsened the outcomes independent of the method of reperfusion.

Clinically, HG was to shown exacerbate the tPA induced cerebral hemorrhage after stroke and our findings from this translational study goes well with the clinical findings. However, in spite of the clinical importance of tPA (as it is the only FDA approved thrombolytic therapy for stroke up till now) and the detrimental effects of the interaction of tPA and HG in AIS settings, only a handful of experimental studies evaluated the impact of the interaction between HG and tPA on stroke outcomes. In 2012, Fan et al showed that the use of tPA in diabetic rats subjected to embolic stroke significantly increased the HT. However, they used a high dose of tPA (10 mg/kg) to induce thrombolysis and restoration of blood flow. The choice of the dose in that study was based on early studies which reported that the rat's fibrinolytic system is 10 times more resistant to the thrombolytic effect of tPA than human [8], and accordingly they used a 10-fold higher dose of tPA (10 mg/kg) than what is used in ischemic stroke patients. In our study, we adopted a novel approach and we used a low dose of tPA of 1 mg/kg which is almost the human dose (0.9 mg/kg). Furthermore, they used a model of streptozotocin induced type 1 diabetes, not acute HG, with very high blood glucose levels (400 mg/dl). Several other groups tried to use low dose of tPA either alone or in combination with other drugs aiming to extend the tPA therapeutic window and reduce the induced HT. In 2010, Zhu et al showed that the combination of low dose tPA (2.5 mg/kg) with annexin A2 (5 mg/kg) in rats subjected to embolic stroke significantly improved CBF, reduced brain infarction and HT [9]. And more recently in 2014, Wang et al showed that a higher dosage regimen of 5 mg/kg tPA combined with 10 mg/kg annexin A2 was able to improve the long term neurological outcomes one month post stroke in normoglycemic animals [10]. However, we are the first to use a low dose that closely mimics the human dose. The rationale behind using tPA

in the suture model was to evaluate the tPA neurotoxic effects irrespective of its recanalization capability. Our results show that infarct size is similar between vehicle and tPA-treated animals suggesting that the "low dose" tPA does not have additional effects on cell death (core formation) as previously reported [11, 12].

Our translational findings showed that the interaction between HG and tPA was detrimental and this effect was independent on the method of reperfusion. Moving forward to the next step, we were interested in investigating the underlying mechanisms through which HG and tPA exacerbate the HT and worsen the outcomes in hyperglycemic stroke, elucidating the role of MMP3.

MMP3 is a zinc endopeptidase that has a broad substrate specificity and can target and degrade all the components of the neurovascular unit and cause disruption of the BBB [13]. It was previously shown that MMP3 plays a critical role in mediating the tPA induced ICH in mice after stroke [14]. However, the role of MMP3 in mediating the HG induced HT, especially with the administration of tPA was not previously studied.

In rats subjected to either suture or thromboembolic occlusion, we found that either HG or tPA alone significantly increased MMP3 activity in the brain and the combination of HG and tPA further increased this activity. Correlating this with our previous findings, we conclude that MMP3 may be mediating the tPA-HG induced HT and vascular injury after stroke. When we tested this in brain vascular endothelial cells (BVECs), we found that tPA significantly increased MMP3 activity in BVECs subjected to either normoxia or hypoxia under normal or high glucose conditions. Opposite to our expectations, there was no further increase in MMP3 activity in cells subjected to hypoxia HG without tPA. This is probably because the insult was not strong enough to trigger such effect. In 2009, Suzuki et al

showed that BVECs derived from mouse brain showed an increase in MMP3 production after being treated with tPA [15]. However, in their study, cells were subjected to 6 h of oxygen/glucose deprivation (OGD) and this effect was not seen when cells were subjected to only 3 h of OGD.

We have shown that HG significantly increased MMP3 activity in the brain and cerebral macrovessels after stroke and this HG induced increase in MMP3 activity was associated with increased HT, edema in the brain and worse functional outcomes. However, the underlying mechanism through which HG activates MMP3 after stroke remained unknown. In 2009, Fujita et al showed that peroxynitrite plays a role in activating MMP3 through tyrosine nitration in the synovial fluid of patients with temporomandibular joint (TMJ) disorders and thus leading to the pathophysiological progression of the internal derangement (ID) of TMJ [16]. This was not previously tested in the brain after stroke. Our immuno-precipitation studies showed that HG increased MMP3 tyrosine nitration in the brains of rats subjected to either suture or thromboembolic occlusion compared to normoglycemic ones. This increase in MMP3 tyrosine nitration corresponded to an increase in MMP3 activity in rats. This was also recapitulated when we tested the same concept in BVECs and we found that cells subjected to high glucose under hypoxic conditions showed significant increase in MMP3 tyrosine nitration. It was previously shown that peroxynitrite levels are elevated in the brain after stroke and HG augmented this effect [17, 18].Thus, we concluded that peroxynitrite mediated tyrosine nitration may be the underlying mechanism through which HG activates MMP3 after stroke.

The HG induced elevation in MMP3 activity in the brain after stroke was associated with significant increase in HT and vascular injury. This injury can be attributed to the

proteolytic ability of MMP3 to degrade the tight junction and extracellular matrix proteins causing disruption of the blood brain barrier and cerebral hemorrhage.

When we examined the spatial expression of MMP3 in the neurovascular unit after stroke, we found that MMP3 was expressed in the neurons, cerebral vessels and surrounding pericytes but not in the astrocytes. In 2007, Suzuki et al showed that MMP3 is expressed in neurons but not endothelial cells after stroke and the administration of tPA selectively induced MMP3 in endothelial cells [14]. They reported also that MMP3 was not seen in the astrocytes after stroke, however, they didn't examine its presence in the pericytes. We found MMP3 expressed in the neurons but not in the astrocytes after stroke in both normoglycemic and hyperglycemic animal without much difference seen between the two groups. However, MMP3 expression pattern was different in the blood vessels of hyperglycemic animals when compared to control ones. In normoglycemic animals, MMP3 was more localized to the pericytes surrounding the blood vessels and was expressed in a spot like form on the vessels body. However, HG induced MMP3 expression along the whole vessels body and surrounding pericytes. This colocalization pattern of MMP3 with cerebral vessels and surrounding pericytes provides a supporting evidence to our findings that MMP3 exacerbates the HT in hyperglycemic stroke. And although we are showing here a pathological role of MMP3 in mediating HT and BBB disruption during the acute phase of the ischemic insult, another group recently published that MMP3 plays a beneficial role during the recovery phase. Yang et al recently showed that MMP3 was expressed in the newly formed blood vessels and pericytes 3 weeks after stroke and that was involved in the neurovascular remodeling and BBB restoration during recovery [19].

This also reflects the dual differential role that MMP3 plays during the acute injury phase versus the late recovery phase.

To further support our findings that MMP3 mediates the HT in hyperglycemic stroke we decided to inhibit MMP3 and check how would this affect stroke outcomes. We used two methods to block MMP3 activity in the brain after stroke. First we employed acute pharmacological inhibition through the use of potent and highly selective MMP3 inhibitor (UK356618). Because we were the first to use the drug in rats, we first performed a series of dose finding studies and finally we selected the dose (15 mg/kg) which achieved 50% or more MMP3 inhibition in activity compared to non-treated animals. Pharmacological inhibition was achieved by a single dose at reperfusion. We found that the drug significantly inhibited MMP3 activity in the brain and cerebrovasculature and that MMP3 inhibition was associated with significant reduction in HT and improvement in functional outcomes. In order to corroborate our findings, we next moved to knock down MMP3 expression by stereotaxic injection of MMP3 shRNA lentiviral particles directly into the lateral ventricles in the brain. We achieved 50% or more reduction in MMP3 expression and this corresponds to a similar degree of reduction in MMP3 activity achieved with the drug inhibition. We found that knocking down MMP3 in the brain significantly reduced HT, vascular injury and improved functional outcomes.

Connecting all the threads together, our findings show that the severity of HG is a determinant of the neurovascular injury after stroke. Mild elevations in blood glucose levels significantly exacerbated the HT and worsened the functional outcomes without increasing the infarct size. This reflects the susceptibility of the vasculature to acute yet mild changes in blood glucose levels, and also reflects how vasculature contribute to the

deterioration in neurobehavioral outcomes without further increase in the neuronal injury. On the other hand, severe HG significantly increased the infarct size compared to control and mild HG groups. We also showed that the combination of HG and tPA significantly increased MMP3 activity in the brain and this corresponded to the exacerbated HT and worsened functional outcomes independent of the method of reperfusion. The significant improvement in the HT and functional outcomes achieved with the pharmacological inhibition and focal knockdown of MMP3 strongly support our hypothesis in demonstrating the deleterious role of MMP3 in mediating HT during the acute phase in hyperglycemic stroke (Fig. 5.1).

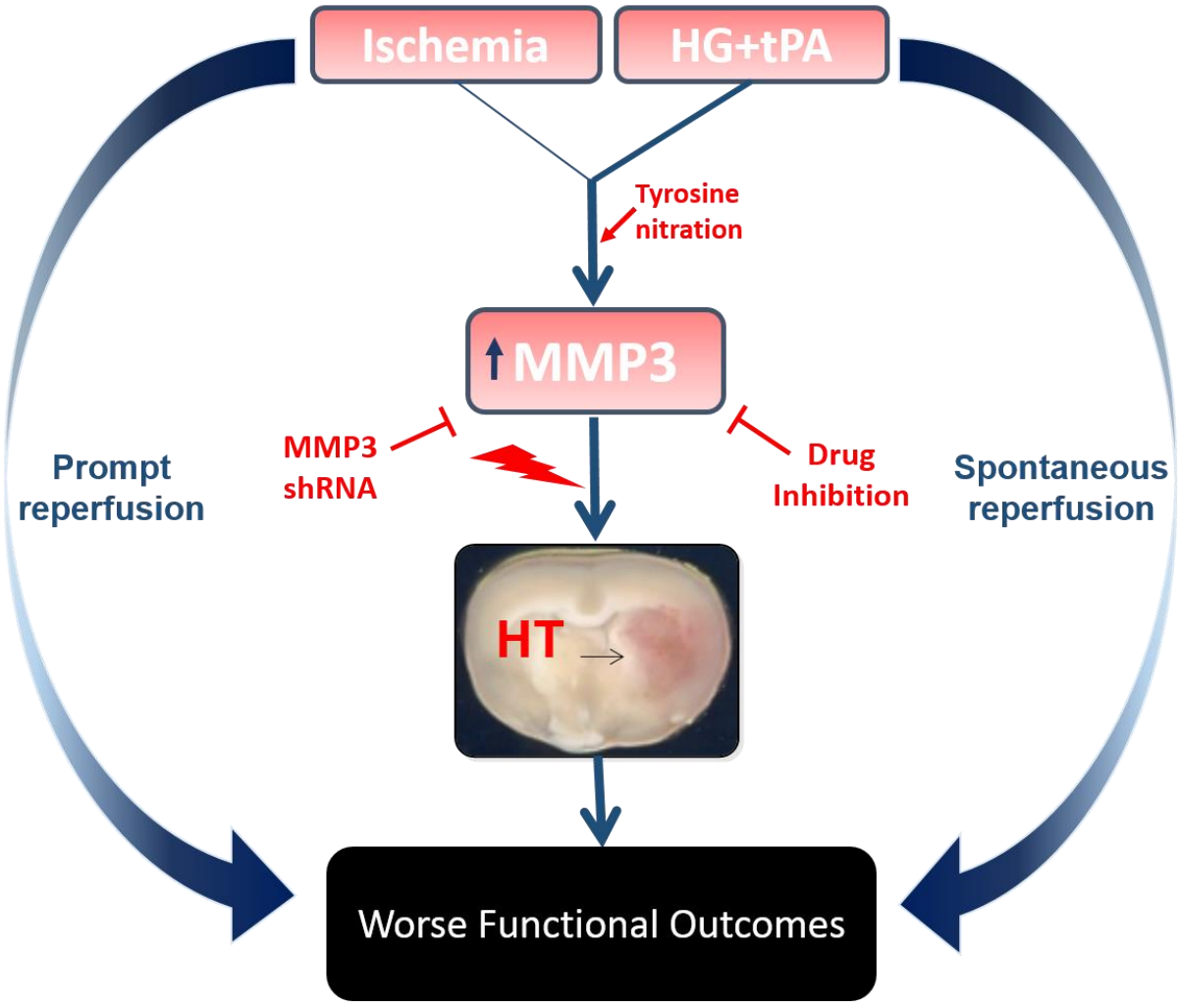
In conclusion, our findings have high translational impact in pointing out MMP3 as a potential therapeutic target for reducing cerebral hemorrhage and improving clinical outcomes in hyperglycemic stroke.

Figure Legends

Figure 5.1. Schematic diagram representing the major findings of our project.

The interaction between HG and tPA after ischemic stroke significantly increased MMP3 activity and this was associated with exacerbated HT and worse functional outcomes, independent of the method of reperfusion. We showed also that tyrosine nitration may be an underlying mechanism causing activation of MMP3 in hyperglycemic stroke. Inhibition of MMP3, either through pharmacological inhibition or through focal knockdown in the brain by MMP3 shRNA, has significantly reduced the HT or improved the functional outcomes.

Figure 5.1. Schematic diagram representing the major findings of our project.



BIBLIOGRAPHY

1. Bruno, A., et al., *Acute blood glucose level and outcome from ischemic stroke. Trial of ORG 10172 in Acute Stroke Treatment (TOAST) Investigators.* Neurology, 1999. **52**(2): p. 280-4.
2. Bruno, A., et al., *Admission glucose level and clinical outcomes in the NINDS rt-PA Stroke Trial.* Neurology, 2002. **59**(5): p. 669-74.
3. Clark, W.M., et al., *The rtPA (alteplase) 0- to 6-hour acute stroke trial, part A (A0276g) : results of a double-blind, placebo-controlled, multicenter study. Thromblytic therapy in acute ischemic stroke study investigators.* Stroke, 2000. **31**(4): p. 811-6.
4. Alvarez-Sabin, J., et al., *Effects of admission hyperglycemia on stroke outcome in reperfused tissue plasminogen activator--treated patients.* Stroke, 2003. **34**(5): p. 1235-41.
5. Poppe, A.Y., et al., *Admission hyperglycemia predicts a worse outcome in stroke patients treated with intravenous thrombolysis.* Diabetes Care, 2009. **32**(4): p. 617-22.
6. Bruno, A., et al., *Treatment of hyperglycemia in ischemic stroke (THIS): a randomized pilot trial.* Stroke, 2008. **39**(2): p. 384-9.
7. Johnston, K.C., et al., *Glucose Regulation in Acute Stroke Patients (GRASP) trial: a randomized pilot trial.* Stroke, 2009. **40**(12): p. 3804-9.
8. Korninger, C. and D. Collen, *Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various animal species in vitro.* Thromb Haemost, 1981. **46**(2): p. 561-5.
9. Zhu, H., et al., *Annexin A2 combined with low-dose tPA improves thrombolytic therapy in a rat model of focal embolic stroke.* J Cereb Blood Flow Metab, 2010. **30**(6): p. 1137-46.
10. Wang, X., et al., *Effects of tissue plasminogen activator and annexin A2 combination therapy on long-term neurological outcomes of rat focal embolic stroke.* Stroke, 2014. **45**(2): p. 619-22.
11. Meng, W., et al., *Effects of tissue type plasminogen activator in embolic versus mechanical models of focal cerebral ischemia in rats.* J Cereb Blood Flow Metab, 1999. **19**(12): p. 1316-21.
12. Lapergue, B., et al., *High-density lipoprotein-based therapy reduces the hemorrhagic complications associated with tissue plasminogen activator treatment in experimental stroke.* Stroke, 2013. **44**(3): p. 699-707.
13. Kim, E.M. and O. Hwang, *Role of matrix metalloproteinase-3 in neurodegeneration.* J Neurochem, 2011. **116**(1): p. 22-32.
14. Suzuki, Y., et al., *Stromelysin-1 (MMP-3) is critical for intracranial bleeding after t-PA treatment of stroke in mice.* J Thromb Haemost, 2007. **5**(8): p. 1732-9.
15. Suzuki, Y., et al., *Tissue-type plasminogen activator (t-PA) induces stromelysin-1 (MMP-3) in endothelial cells through activation of lipoprotein receptor-related protein.* Blood, 2009. **114**(15): p. 3352-8.
16. Fujita, H., et al., *MMP-3 activation is a hallmark indicating an early change in TMJ disorders, and is related to nitration.* Int J Oral Maxillofac Surg, 2009. **38**(1): p. 70-8.

17. Bemeur, C., L. Ste-Marie, and J. Montgomery, *Increased oxidative stress during hyperglycemic cerebral ischemia.* Neurochem Int, 2007. **50**(7-8): p. 890-904.
18. Ste-Marie, L., et al., *Immunohistochemical detection of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase following hyperglycemic focal cerebral ischemia.* Brain Res, 2001. **918**(1-2): p. 10-9.
19. Yang, Y., et al., *Early inhibition of MMP activity in ischemic rat brain promotes expression of tight junction proteins and angiogenesis during recovery.* J Cereb Blood Flow Metab, 2013. **33**(7): p. 1104-14.