EFFECTS OF HYPERGLYCEMIA ON CEREBROVASCULAR STRUCTURE, FUNCTION AND ISCHEMIC BRAIN INJURY

by

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(Under the Direction of Adviye Ergul)

ABSTRACT

Statement: Admission hyperglycemia impacts ischemic stroke deleteriously but the relative role of acute hyperglycemia (HG) versus diabetes in the pathogenesis of this poor outcome is not clear. We have shown that middle cerebral artery occlusion (MCAO) causes greater hemorrhagic transformation (HT) in diabetic Goto-Kakizaki (GK) rats, a model with increased cerebrovascular matrix metalloprotease (MMP) activity and tortuosity.

Objectives: 1) Determining the effect of HG on neurovascular outcomes of stroke in control versus diabetes, 2) Determining whether diabetes-induced cerebrovascular remodeling is MMP-dependant and 3) To show that prevention of vascular remodeling by glycemic control or MMP inhibition reduces HT in diabetic stroke.

Methods: HG was achieved by glucose infusion before MCAO in control Wistar and mildly diabetic GK rats. Following 3 h MCAO/21 h reperfusion, we measured infarct size, HT frequency, excess hemoglobin, neurobehavioral outcome and baseline plasma and MCA MMP activity. Following chronic treatment with metformin or minocycline in a different cohort, we measured baseline cerebrovascular remodeling indices, MCA MMP activity and infarct size and HT after MCAO.

Results: Infarct size was significantly smaller in diabetes. HG increased neuronal damage in diabetes but not in control. HT frequency and hemoglobin were significantly higher in diabetes. HG augmented HT in control but not in diabetes. Baseline plasma MMP-9 activity was significantly higher in diabetes. HG increased MMP-9 activity in control and diabetes. Neurological deficit was greater in diabetes. All remodeling markers including MMP-9 activity were increased in diabetes and both metformin and minocycline prevented these changes. Infarct size was smaller in minocycline-treated animals and both metformin and minocycline reduced incidence and severity of HT.

Conclusions: HG worsens outcome from ischemic stroke and induces HT in control rats. A further glycemic increase in diabetes does not worsen HT suggesting baseline vascular damage. Higher basal plasma MMP-9 levels in diabetes are associated with higher HT. Since 24 h levels do not correlate with HT, earlier time points merit investigation. Diabetes-mediated stimulation of cerebrovascular MMP-9 activity promotes cerebrovascular remodeling and greater HT in diabetes. Metformin and minocycline offer vascular protection for diabetes patients who are at a 4 to 6-fold higher risk for stroke.

INDEX WORDS: Diabetes, hyperglycemia, acute ischemic stroke, MMPs, MMP-9, hemorrhagic transformation, vascular protection, metformin and minocycline.

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DEDICATION

To my father *Dr Mahmoud Elgebaly* for whom I have done this and whom I love more than anyone, to my mentor *Dr Adviye Ergul* who had faith in me all these years and gave me her unconditional support, knowledge and time and to my *mother*.

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Finally as I have come to learn from my life experience, I must acknowledge the inner source of my strength, *God*, the ultimate loving father.

Science is the only true guide in life "Mustafa Kemal Atatürk".

From error to error, one discovers the entire truth "Sigmund Freud".

Let the beauty of what you love be what you do "Rumi-Mevlana".

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Problem statement and objectives

Every 45 seconds in the USA, there is a new stroke with a total of 795,000 patients yearly, out of which 83% are acute ischemic strokes (AIS). Type-2 diabetes increases the relative risk and poor outcome of stroke 2 to 6-fold. Moreover, almost 50% of AIS patients present with admission hyperglycemia that can be independent of diabetes and this is positively associated with longer hospitalization, a 3-fold increase in mortality and poor functional recovery. However, the impact of acute hyperglycemia vs diabetes on the neurovascular unit and the mechanisms by which either may predispose AIS patients to a worsened outcome are yet to be identified.

Acutely during ischemia reperfusion injury in the brain, MMP-2 and MMP-9 mediate blood brain barrier (BBB) breakdown leading to edema as well as complicating AIS by causing hemorrhagic transformation (HT) secondary to ischemia. Clinically, admission hyperglycemia and a history of diabetes predict HT, which is a major limitation in the administration of intravenous tissue plasminogen activator (t-PA), the only FDA-approved therapy for AIS. Our laboratory has recently shown that the Goto-Kakizaki (GK) rat model of type-2 diabetes displays increased basal cerebrovascular MMP activity and remodeling prior to stroke. In addition, we have shown that following transient ischemia induced by 3 h MCAO and 24 h reperfusion, GK rats display a dramatic increase in HT yet

infarct size is smaller compared to normoglycemic control rats. These 2 interesting findings strongly suggest that acute hyperglycemia and diabetes may cause ischemic damage by different mechanisms and thus form the basis of this proposal to determine the effects of acute vs chronic hyperglycemia on cerebrovascular therapeutic targets identified in stroke and on stroke outcome. ***The driving hypothesis*** is that MMP-mediated cerebrovascular remodeling in type-2 diabetes contributes to increased occurrence of HT following ischemic injury whereas acute hyperglycemia during stroke augments neurovascular damage. It is also hypothesized that tight control of blood glucose or inhibition of MMPs reduces HT in diabetes (Fig. 1.1).

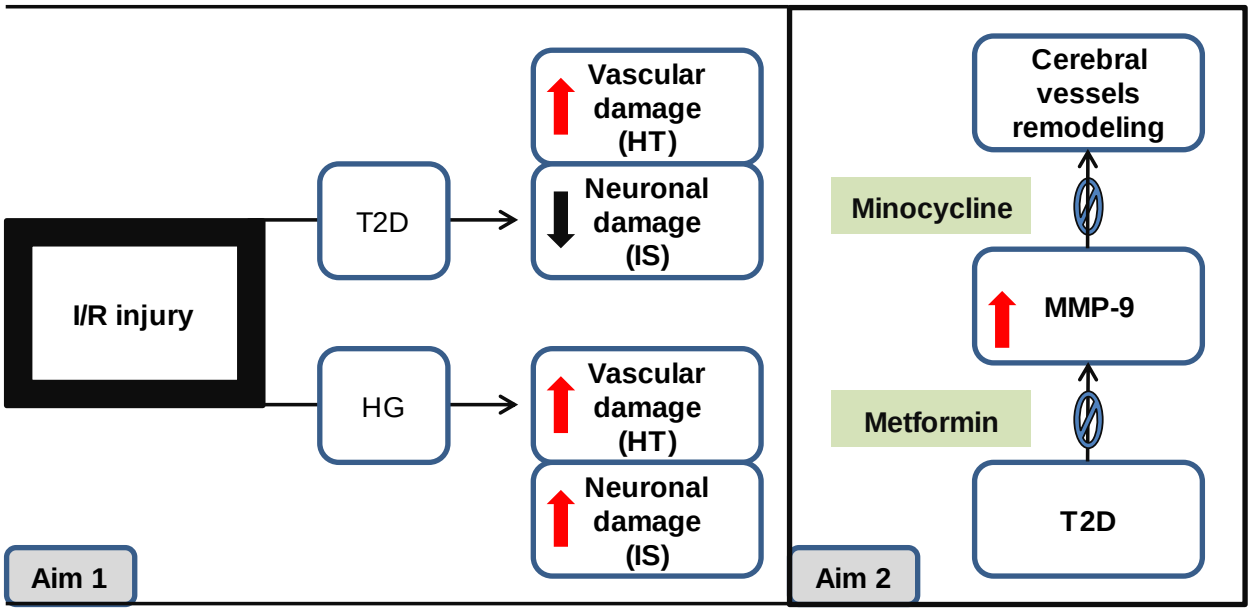

Fig. 1.1. **Schematic representation of hypotheses and experimental design.** I/R: ischemia/reperfusion, T2D: type-2 diabetes, HG: acute hyperglycemia, HT: hemorrhagic transformation and IS: infarct size.

Aim 1

To determine the relative contribution of acute hyperglycemia vs chronic hyperglycemia (diabetes) to vascular (HT) and neuronal (infarct) damage in ischemia/reperfusion injury.

We will determine:

1. Infarct size, edema, HT and neurological outcome in normoglycemic and acutely hyperglycemic (glucose injection) control Wistar as well as in diabetic, euglycemic (metformin-treated) and acutely hyperglycemic GK (diabetes + glucose injection) rats using 3 h MCAO/24 h reperfusion model.

Completion of this aim will determine to what extent acute hyperglycemia and diabetes exacerbate neuronal and/or vascular damage.

In order to evaluate the use of plasma MMPs as prognostic markers of stroke outcome in diabetes, we will determine:

2. Plasma MMP activity in control and diabetic animals subjected to MCAO.

Aim 2

To determine the extent to which diabetes-induced upregulation of MMP activity promotes cerebrovascular remodeling resulting in HT following ischemia/reperfusion injury.

We will evaluate:

1. Vascular remodeling indices tortuosity index (TI) and MCA structure in vehicle, metformin-treated (glycemic control) and minocycline-treated (MMP inhibition) GK and control rats before ischemia/reperfusion injury (I/R).
2. MMPs expression and activity, infarct size, edema, HT and neurological outcome in the same groups as above before and after (I/R) injury.

By completing this aim, we will identify potential mechanisms contributing to preexisting cerebrovascular disease and/or HT in I/R injury in diabetes.

Despite extensive research, with the exception of t-PA, all treatment strategies identified in otherwise healthy animal models of stroke failed in clinical stroke research. Given the high incidence of diabetes or admission hyperglycemia in AIS patients, therapeutic strategies should take into account the impact of existing vascular disease on stroke pathophysiology. Therefore, this work, focusing on the regulation of cerebrovascular structure in diabetes and acute hyperglycemia, represents an innovative approach to identify novel therapeutic targets for this high risk diabetic population.

Literature review and project rationale discussion

Type-2 diabetes

Type-2 diabetes represents 90-95% [1-4] of all diagnosed cases of diabetes. The disease historically known as adult-onset diabetes mellitus has been diagnosed in an alarming fashion in adolescents and youngsters below 20 years old over the past 20 years [1, 5]. The metabolic disorder is of an unknown etiology. However, both combined genetic and environmental risk factors have been long established for predisposing individual to diabetes. They include family history, sedentary lifestyle, obesity, hypertension, dyslipidemia, ethnicity and a history of gestational diabetes mellitus [6-10]. Once type-2 diabetes develops, it can not be reversed. Yet, it can be maintained through controlling the disease hallmark namely hyperglycemia, thus reducing the disease complications. However, if prediabetes is diagnosed early using impaired glucose tolerance and impaired fasting glucose tests, type-2 diabetes can be prevented by proper diet and modifiable risk factors strict management. As a result of the inability of pancreas to meet the cellular needs for insulin secretion and insulin resistance, hyperglycemia ensues and is responsible for the major complications of diabetes. If left uncontrolled, type-2 diabetes patients are at risk for cardiovascular diseases including myocardial infarction, stroke and atherosclerosis as well as classic pathophysiology of diabetes such as retinopathy, nephropathy and neuropathy [11-13].

AIS

Seven hundred ninety five thousand patients suffer a new or recurrent stroke in USA every year [1, 14]. Stroke or brain infarct is the third cause of death and a leading cause of functional disability among its survivors. It is classified as either 1) Ischemic (83% AIS) due to a clogged artery or 2) Hemorrhagic (17%) as a result of arterial rupture. In AIS, the neurovascular unit components of brain, both vessels and neurons, are deprived of oxygen and nutrients. The infarct area is divided into 2 regions:

1. The core which is severely damaged and is a dead tissue.
2. The penumbra which is the area at risk of death and that could be salvaged within a limited time if reperfusion is adequately restored.

With mainly a single FDA-approved pharmacologic treatment for AIS and with the failure of numerous therapeutic modalities in clinical trials, strategies aimed at effective primary prevention are important in managing AIS. Diabetes has been considered one of the well documented risk factors and independent predictors of AIS and serves potentially as a good target for primary prevention. Currently, the guidelines show beneficial effects for controlling diabetes on microvascular complications especially in hypertensive patients. However, the evidence for reducing stroke risk is still lacking.

Type-2 diabetes impact on stroke as a classic risk factor

Over 1,000,000 stroke survivors suffer persistent neurological deficit making it the leading cause of long term and serious disability [1, 15, 16]. Type-2 diabetes is a rapidly growing modifiable risk factor in stroke management.

Prevalence of type-2 diabetes among US adults has increased by over 50% in the past decade, currently representing 7% of the population- 21 million Americans [1]. At least 65% of type-2 diabetes patients die of a heart disease or stroke [17, 18]. Ischemic stroke relative risk in diabetics is 2-6 fold higher than normal population [6], with as many as 20% of strokes attributable to type-2 diabetes [11, 19]. Non-diabetics with insulin resistance, the cardinal feature of the metabolic syndrome, also have higher risks for cerebral infarction [20].

Type-2 diabetes and hyperglycemia worsen stroke outcomes

Type-2 diabetes patients have both higher incidences of stroke and worse outcomes. One week, 1 and 3 months mortality following stroke is higher and survivors exhibit more severe neurological deficits and disability [21, 22]. Early in-hospital recovery is worse in type-2 diabetes patients with more confinement to bed. In addition, type-2 diabetes and hyperglycemia are early predictors of neurological deterioration following AIS [23, 24]. Bruno et al. reported worse neurological outcome associated with higher admission hyperglycemia [25-28]. Type-2 diabetes is a risk factor for AIS independent of other cardiovascular confounders e.g. dyslipidemia and hypertension [19]. In the literature there is little known regarding the relative role of acute vs chronic hyperglycemia in the pathogenesis of this poor outcome [29, 30]. A recent meta-analysis showed a differential effect of acute hyperglycemia independent of type-2 diabetes on stroke outcomes [31]. Acute hyperglycemia impact was worse and resulted in 3 times higher rates of one month mortality compared to a 2 fold increase in case of diabetes. The main events involved in hyperglycemia-induced neurovascular damage

during ischemic stroke include: rapidly growing infarction, edema, increased intracranial pressure and HT.

The NINDS rt-PA (National Institute of Neurological Disorders, recombinant tissue Plasminogen Activator) stroke trial showed that admission hyperglycemia is a significant predictor of poor clinical outcome and HT thus suggesting that HT of infarct may be an important factor behind neurological deterioration following AIS [28]. Normoglycemia and no history of type-2 diabetes are predictors of good outcomes. On the other hand, admission hyperglycemia and a history of type-2 diabetes are predictors of t-PA-induced HT [32, 33]. Odds of HT in presence of hyperglycemia after t-PA treatment appear highest with early re-canalization, antagonizing potential benefits from reperfusion and highlighting the detrimental effect of hyperglycemia in worsening I/R injury [34, 35]. GIST-UK (Glucose Insulin Stroke Trial – United Kingdom) is so far the biggest trial to show that tight control of hyperglycemia should improve AIS outcomes [36]. Unfortunately, the early benefits seen within the first week, were later lost with no difference between the control and treatment group. Bruno et al. tested the feasibility of achieving target blood glucose levels of < 130 mg/dl by IV insulin infusion during acute AIS in a small number of type-2 diabetes patients [37]. Target blood glucose levels were safely achieved yet larger clinical trials are needed to assess effects of controlling glucose on stroke outcomes.

Type-2 diabetes and hyperglycemia impact on AIS in experimental models

The adverse effect of hyperglycemia on cerebral ischemic damage is generally well accepted. Myers and Yamaguchi were the first to report that acute hyperglycemia augmented neuronal injury after cardiac arrest-induced global ischemia in monkeys [38]. In the following years, the deleterious effect of acute hyperglycemia on brain injury after global ischemia was confirmed in other studies [39, 40]. Natale et al. demonstrated increased mortality in dogs with moderate hyperglycemia (18 ± 0.9 mmol/L) following global ischemia and this was associated with increased lactate levels in the cortex [41].

In permanent focal ischemia models, most authors indicate that hyperglycemia increases ischemic damage. De Courten-Myers et al. reported increased infarct size in both brief and prolonged hyperglycemia states after permanent MCAO in cats where acute hyperglycemia was induced by glucose injection [42]. Compared to normoglycemia, acutely hyperglycemic cats had a 3-fold increase in hemispheric infarct volume. However, there are also other studies that showed contrary results. Nedergaard et al. reported that compared with normoglycemia, the infarct volume was decreased in hypoglycemic rats, unaltered in acute diabetes induced by single streptozotocin (STZ) injection 2 days before MCAO and increased in chronic diabetes induced by STZ injection 4 months before MCAO [43, 44]. In another study by the same group, the researchers found that the cortical glucose metabolism remained normal and there was no neuronal loss in the penumbra of hyperglycemic rats after MCAO. These results indicated that hyperglycemia might protect against neuronal injury in the areas next to the infarct. In a study with photochemically induced permanent cerebral ischemia, acute severe hyperglycemia induced by glucose injection (range 15-34

mmol/L) was reported to result in smaller infarct volume compared with normoglycemic animals (range 4-10 mmol/L) [45]. Similar results have been reported in rabbits and cats [46, 47].

The results from reversible focal ischemia models are also variable. Most studies reported increased brain injury in hyperglycemic animals after reperfusion [48-50]. When hyperglycemia was induced acutely in spontaneously hypertensive rats, there was no effect of hyperglycemia on infarcts [51]. De Courten-Myers et al. demonstrated that acutely hyperglycemic cats had a 7-fold increased death rate due to hemispheric edema after transient MCAO, whereas there was no difference in infarct volume between control and acutely hyperglycemic animals [48]. By using magnetic resonance imaging (MRI) techniques, Quast et al. determined the effect of preexisting hyperglycemia on I/R injury in acute hyperglycemia induced by STZ injection 2 days prior to induction of stroke [36]. Larger lesion size and lower hemispheric blood volume were found in hyperglycemic animals after temporary, but not permanent, ischemia. In recent studies, Ennis and Keep have reported marked BBB disruption in intraperitoneal glucose injection-induced mild (5.5-11 mmol/L) and transient severe (>20 mmol/L) hyperglycemia after temporary and permanent occlusion [52]. Similar results were shown in a recent study by Kamada et al. [53]. In STZ-induced acute hyperglycemia, they reported increased edema volume and Evans blue leakage after 60 min MCAO. While there are a few studies that report otherwise, as summarized above most of these past studies point to greater ischemic damage in animals made hyperglycemic acutely by glucose injection or STZ injection 2-3 days prior to ischemic injury. I/R injury not only results in neuronal damage but also vascular damage leading to HT. This phenomenon

is distinct from primary intracerebral hemorrhage and develops secondary to prolonged brain ischemia. Hyperglycemia causes increased HT in reversible MCAO models indicating that reperfusion injury contributes to the development of this complication [48, 54]. Even the acute hyperglycemia induced by anesthesia worsens HT and induces larger infarct size after reperfusion [55].

Data on the effect of type-2 diabetes, or what could be perceived as long term hyperglycemia, are limited. Vannucci et al. have reported increased edema and infarct size after hypoxic-ischemic injury in db/db mice compared to non-diabetic animals. In this model blood glucose levels were >22 mmol/L and hypoxia-ischemia was induced by first ligating the carotid artery and 3 h later exposing the animals to 8% oxygen/92% nitrogen gas mixture for 15-30 min [56]. Interestingly, an earlier study by Warner et al. reported that acutely hyperglycemic but nondiabetic rats were more vulnerable to global ischemia despite similar levels of glycemia suggesting some mechanism of protection or adaptive response in diabetes [57]. Again, in this study blood glucose levels were higher than normally seen in type-2 diabetes patients and it employed a global ischemia method. Recently we reported a smaller volume and characteristic subcortical localization of infarcts after temporary MCAO in GK rats, a lean model of type-2 diabetes with moderate levels of glycemia [58]. In our studies, the duration of diabetes was 4-6 weeks and average blood glucose was 10-12 mmol/L. Moreover, in all diabetic animals there was HT and increased edema after transient [58] but not permanent ischemia [59].

MMPs as mediators of HT and biomarkers after AIS

BBB integrity is maintained by tight junctions between the neurovascular unit endothelial cells, an intact basal lamina and extracellular matrix; leading to the creation of a barrier o high selective permeability. Reperfusion of ischemic brain tissue results in the breakdown of the BBB due to loss of basal lamina integrity and dissolution of the extracellular matrix leading to brain edema [60]. MMPs are a class of zinc-dependent endopeptidases physiologically involved in degrading and remodeling extracellular matrix components. Regulated on different levels, they are secreted as prozymes requiring cleavage of propeptide region for activation. MMPs implicated role in BBB breakdown and the resulting extravasation of red blood cells into cerebral tissue is supported by several sources. Following MCAO in a non-human primate, MMP-2 levels increased early (1 h) and correlated with the extent of neuronal injury while MMP-9 expression was increased only in subjects with HT [61]. MMP-9 knockout mice were strongly protected against ischemic injury following transient focal ischemia with reduction of infarct size and reduced degradation of BBB components [62]. Clinically, levels of plasma MMP-9 are predictive of HT following AIS [63-66]. MMP-9 appears to mediate HT after t-PA administration, reducing the benefit of early reperfusion [67]. The mechanisms by which t-PA is postulated to increase MMP-9 levels are both indirect, related to free radicals induction during reperfusion injury, and direct, by binding to the low-density lipoprotein receptor-related protein [68]. Clinically brain MMP-9 basal levels are undetectable but following a brain injury such as stroke, they are upregulated. Acutely, there is a biphasic BBB opening exacerbating brain edema and hemorrhage. High levels of MMPs may be used as surrogate markers for deleterious events.

However, it has been shown that later MMPs play a pivotal role in repairing the damaged neurovascular unit. In order to achieve adequate neuroprotection, inhibiting MMPs should be limited within the acute phase i.e. up to 3 days. There is evidence that later (7 days) inhibition may actually worsen stroke outcomes due to the antagonism of MMP-dependant repair remodeling.

t-PA, bleeding and MMPs

Despite the beneficial effects of early reperfusion after t-PA use, its benefits remain limited for two main reasons. First of all, of all patients ischemic stroke patients, only 3-5% of qualified patients receive t-PA mostly due to arriving too late [28]. Secondly , the adverse drug reaction, mainly bleeding, occurs in about 15% of patients receiving t-PA [69]. Therefore, it is crucial to be able to find specific patient populations who are at higher risks for t-PA induced hemorrhagic conversion through studying potential biomarkers as MMP-9. It is also important to develop strategies to safely administer t-PA and expand the therapeutic window. One approach would be to block the MMP-mediated HT without affecting the thrombolytic activity of t-PA.

Minocycline as a MMP-9 inhibitor and neuroprotective drug

Minocycline is a second generation tetracycline mainly used as a classic antimicrobial agent. It is highly lipophilic and thus crosses the BBB easily to exert its action [70]. It has been shown to be effective in reducing intracerebral hemorrhage in a thrombotic stroke model [71]. In another *in vitro* study it has been shown to inhibit MMP-9 [72] and it also exhibits anti-inflammatory action [73]. Although it is a non-specific

MMP-9 inhibitor, its use has many advantages:

1. An established safety profile based on its classical use as an antimicrobial agent. There is an ongoing phase-I dose finding trial (MINO, Minocycline to Improve Neurologic Outcome) to investigate the neuroprotective effect of minocycline in AIS patients as MMPs inhibitor. Phase-III is currently being planned.
2. Machado et al. showed that even when administered after thrombolysis in AIS, minocycline was still able to inhibit the activity of MMP-9. This finding gives the drug a value in clinical application due to the extended time window of this mechanism of action [72].
3. When its class-equivalent tetracycline was used clinically, it successfully decreased MMP-9 in abdominal aortic aneurysm and carotid plague patients [74].
4. Experimentally MMPs were shown to be inhibited by minocycline in a dose-dependent manner [75-77]. A proposed mechanism of action was through the divalent metal chelating ability of tetracyclines which in turn will cause structural instability in MMPs which are Zinc-dependant [78-81].

Microvascular and macrovascular complications of diabetes

Hyperglycemia which is the hallmark of type-2 diabetes represents an important therapeutic target. Controlling hyperglycemia ultimately leads to protecting the vasculature and in turn reduces both mortality and morbidity of type-2 diabetes. Type-2 diabetes has two well documented types of vascular complications, microvascular (retinopathy, neuropathy and nephropathy) and macrovascular (stroke, coronary artery disease and peripheral arterial disease). Controlling hyperglycemia is well known to

improve the microvascular complications of type-2 diabetes [20]. However, more clinical trials are needed to conclusively show the long term benefits on macrovascular events. The most recent major prospective randomized controlled clinical trial showed controversial results regarding “tight glycemic” control outcome on macrovascular events. These trials aimed at target levels of HbA_{1C} of < 6% that is more stringent than the usually recommended target level in anticipation of better macrovascular outcomes with such tighter glycemic control [26, 82-87].

ACCORD (Action to Control Cardiovascular Risk in Diabetes) was prematurely stopped due to the increased mortalities in the tight control arm. However, ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation) and VADT (Veteran Affairs Diabetes Trial) did not replicate the same findings [21-23]. The precise reason for this unexpected result is not clear yet. Post-hoc analyses looked at reasons such as stage of cardiovascular condition at baseline, speed of glycemic control achieved, combination of drugs used and the reasons for hypoglycemic episodes but no conclusion was reached. However, ADVANCE showed reduction in nephropathy, a microvascular event, which in turn is a long term risk factor for cardiovascular disease [24].

In the latest statement released by the American Diabetes Association the consensus was that despite the non-significant reduction in macrovascular events in these 3 trials, the long term follow-up of DCCT (Diabetes Control and Complications Trial) and UKPDS (United Kingdom Prospective Diabetes Trial) showed that early control with target HbA_{1C} around 7% is associated with long term reduction in macrovascular events. In the future better and more defined criteria for randomization,

well defined primary end points with clinical significance and treating other cardiovascular diseases still need to be addressed [26].

While mechanisms may vary, both macro and microvascular complications associated with diabetes present with changes in vascular structure and function [11, 88, 89]. Diabetes promotes vasculopathy including proliferation of vascular smooth muscle cells (VSMC), degeneration of endothelial cells and pericytes, thickening of the capillary basement membrane and an increase in aggregation and adhesion of platelets to endothelium [90]. These changes are rarely seen in acute hyperglycemia. Vascular remodeling in diabetes is generally hypertrophic as a result of wall growth (increased collagen deposition and or VSMC hypertrophy/hyperplasia) as opposed to the eutrophic remodeling seen in hypertension due to rearrangements of VSMC around the lumen [91-95]. Consequently, the media thickens progressively, the lumen narrows and the M/L ratio increases [96]. Different mechanisms were proposed for type-2 diabetes-induced remodeling including contribution of insulin and insulin like growth factor, increased intraluminal wall stress due to impaired myogenic tone, increased matrix collagen deposition leading to narrower lumen and upregulation of neuro-humoral factors (MMPs, Endothelin-1 and Vascular Endothelial Growth Factor) [97-99]. The resulting angiopathy ultimately affects both microvessel and macrovessel function and may help explain the increased clinical manifestation of cardiovascular events which are the predominant reason behind patients higher rates of mortality.

We have shown that short term diabetes (4 weeks) is associated with increased tortuosity index (TI) of pial vessels [58]. At this point, there is no change in the MCA wall and lumen thickness but MMP activity is already increased. Changes in vascular

structure may influence vascular tone and integrity which ultimately affect cerebral blood flow and the magnitude of I/R injury. We have recently demonstrated that animals that display increased TI and MMP activity after a short duration of diabetes present with smaller infarcts but greater HT providing compelling evidence that preexisting vascular disease may have a differential effect on hyperglycemic ischemic injury.

Type-2 diabetes upregulates MMPs

Enhanced MMP-9 expression and activity under conditions of elevated glucose is well documented. This was shown in both plasma and vascular tissue from two different rodent models of type-2 diabetes, as well as bovine aortic endothelial cells and human monocyte-derived macrophages incubated under hyperglycemic conditions [100, 101]. In addition, although “healthy”, newly diagnosed diabetic patients do not have elevated plasma MMP-9 levels, plasma taken from diabetics with peripheral arterial disease shows increased MMP-9 levels and higher zymographic activity [102]. MMPs play important roles in reshaping vascular wall in health and disease. MMPs expression is induced by proinflammatory cytokines and growth factors. Our group has shown for example that the ET-1 system upregulation in type-2 diabetes cerebral vessels precedes MMP system dysregulation and ultimately leading to M/L thickening [92]. In microvessels, hyperglycemia upregulates MMP-9 in the retina and increases vascular permeability, the basement membrane degrades and tight junctions are disrupted [103]. Thus regulation of MMPs is critical for both diabetes and stroke pathophysiology.

Cerebrovascular function in acute hyperglycemia and diabetes

Diabetes impairs vasorelaxation and augments vasoconstriction in many vascular beds [104-106]. Our group has recently shown that basilar arteries present with significantly diminished relaxation in GK rats [107]. Myogenic tone (pressure-induced), endothelial and neuronal factors all contribute to the regulation of vessel caliber and very small changes in vessel caliber can have a very significant effect on cerebral blood flow. The cerebral circulation has autoregulatory properties to maintain constant blood flow. Thus, myogenic tone is critical for controlling cerebral blood flow under normal conditions and more so in I/R injury. Basal myogenic reactivity is defined as the ability of VSMC to constrict as the pressure increases to keep the blood flow constant. In the cerebral circulation, this autoregulation is functional within pressure ranges of 40-140 mmHg [108]. Cipolla et al. first reported that preexisting myogenic tone of posterior cerebral arteries was decreased in association to increased glucose concentration [109]. However, another study done by Zimmermann et al. demonstrated greater membrane potential depolarization and constriction of MCAs from diabetic female Sprague-Dawley rats compared with controls [110]. A recent study in the BBZDR/Wor rat model of type-2 diabetes reported that posterior cerebral artery tone is also enhanced [111]. Since regulation of cerebral blood flow is very important for stroke pathophysiology, deleterious effects of type-2 diabetes on cerebrovascular tone might have a negative impact on AIS injury. It is therefore important to investigate the relation between type-2 diabetes-induced vascular pathology, effects of type-2 diabetes on cerebrovascular tone, how these changes might impact AIS injury and how effective glycemic control might be in ameliorating the injury.

The gap in our current knowledge

In summary, our knowledge about the regulation of cerebrovascular reactivity and tone in hyperglycemic and diabetic stroke models is very limited. Also, we still lack a clear understanding of the mechanisms behind loss of the BBB integrity and vascular structure abnormalities when diabetes and/or hyperglycemia are superimposed on AIS.

CHAPTER 2

NEUROVASCULAR OUTCOMES IN ACUTE HYPERGLYCEMIA AND DIABETES: A COMPARATIVE ANALYSIS IN EXPERIMENTAL STROKE

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Abstract

Background: Admission hyperglycemia impacts ischemic stroke deleteriously but the relative role of acute hyperglycemia (HG) versus diabetes in this poor outcome pathogenesis is not clear.

Purpose: Determining the effect of HG on neurovascular outcomes of stroke under control and diabetic conditions.

Methods: Moderate HG (140-200 mg/dl) was achieved by glucose injection before middle cerebral artery occlusion (MCAO) in control Wistar and diabetic Goto-Kakizaki (GK) rats. Following 3 h MCAO/21 h reperfusion, we measured infarct size, hemorrhagic transformation (HT) frequency and excess hemoglobin after assessment of neurobehavioral outcome. We also measured plasma and MCA matrix metalloprotease (MMP) activity.

Results: Infarct size was significantly smaller in diabetic rats. Moderate HG increased neuronal damage in diabetic but not in control rats. HT frequency and hemoglobin were significantly higher in diabetic rats. HG augmented vascular damage in control rats and had no additional effect on bleeding in diabetic rats. Baseline plasma MMP-9 activity was significantly higher in diabetic rats. HG increased MMP-9 activity in control and diabetic rats. Neurological deficit was greater in diabetic rats and was worsened by HG.

Conclusions: Moderate hyperglycemia worsens outcome from ischemic stroke. While moderate HG does not increase infarct size in control rats, it induces HT. A further elevation in blood glucose in diabetic rats does not worsen HT suggesting

baseline vascular damage in diabetic rats. Higher basal MMP-9 levels in diabetic rats are associated with higher HT. However, 24 h plasma MMP-9 levels do not correlate with HT and temporal profile with earlier time points merit investigation.

Introduction

Ischemic stroke is a leading cause of death and disability in the United States and diabetes is the most rapidly increasing risk factor for stroke [112]. Stroke risk in patients with diabetes is 2-6 fold higher than age-matched controls [112, 113]. The short and long term functional outcomes are worse and mortality is greater in stroke patients with diabetes as compared to the non-diabetic population [114, 115]. In addition to increased risk for stroke with diabetes, acute hyperglycemia (HG) that can develop as a stress response exacerbates stroke [28, 116, 117]. However, it is not clear whether there is a difference between the effect of HG vs diabetes on clinical stroke outcomes [31]. Target blood glucose levels to reduce ischemic brain damage and improve stroke outcomes may be different for hyperglycemic versus diabetic patients. This is clinically important as the current stroke guidelines emphasize the need for randomized controlled trials to determine the best practice for managing hyperglycemia [118]. The GIST-UK randomized clinical trial [36] enrolled predominantly patients without diabetes (83%). Although treatment with insulin achieved target blood glucose levels, there was no difference in stroke outcomes. Results from the Treatment of Hyperglycemia in Ischemic Stroke (THIS) [37] and the Glucose Regulation in Acute Stroke Patients (GRASP) [119] pilot trials, both of which enrolled mainly diabetic patients (>50%), have suggested a favorable outcome in acute stroke patients rapidly treated for

hyperglycemia. While these results need to be confirmed in larger trials, they also highlight the importance of targeting the right patients and the right blood glucose range in clinical trials.

Previous preclinical studies on diabetic and hyperglycemic ischemic brain injury do not provide sufficient information as most studies employed HG induced by either glucose injection or streptozotocin (STZ) injection 2-3 days prior to stroke with very high blood glucose levels [42, 48, 53, 54, 120, 121]. However, recent clinical evidence suggests that blood glucose levels in stroke patients that present with hyperglycemia at admission range between 140-200 mg/dl [31, 37, 122]. Understanding the mechanisms behind diabetes versus HG-dependant stroke pathology in animal models that closely represent the clinical condition is paramount and can provide insight to improve current preventive and therapeutic interventions.

Matrix metalloproteases (MMP) play an important role in vascular remodeling as well as stroke pathophysiology [61, 62, 65, 123-127]. We previously reported that augmented cerebrovascular MMP-2 and MMP-9 activity in the Goto-Kakizaki (GK) model of diabetes is associated with enhanced remodeling [92] and increased hemorrhagic transformation (HT) following ischemic stroke [58]. Clinically, plasma MMP-9 levels are predictive of HT following acute ischemic stroke [64, 65]. However, regulation of cerebrovascular and plasma MMP-9 levels in hyperglycemic and diabetic models and the subsequent effects on stroke pathology remain unknown.

Building upon these previous findings, this study was designed to address the questions: 1. How do moderate acute hyperglycemia and diabetes affect neurovascular damage and stroke outcome?, 2. Does a further acute elevation in blood glucose at the

time of ischemia exacerbate neuronal and vascular injury in diabetes? and 3. How do HG and diabetes influence vascular and plasma MMP-9 following ischemic stroke?.

Materials and methods

Animal models

All protocols were approved by the institutional care and use committee (IACUC) of the Medical College of Georgia. Male Wistar and Goto-Kakizaki (GK) rats were purchased from Harlan (Indianapolis, IN) and Taconic (Hudson, NY) Laboratories, respectively, and ranged in weight 260-310 g. Metformin, titrated from 150-300 mg/Kg/day based on blood glucose levels (MP Biomedicals, catalogue# 157805), was given to maintain euglycemia in one group of GK rats. Metformin treatment was initiated at the onset of diabetes in GK rats and was given in drinking water artificially sweetened by a non-caloric sweetener for 4 weeks immediately preceding the study. Acute HG was achieved by 3 ml IP glucose injection (300 mmol) 20-30 minutes before middle cerebral artery occlusion (MCAO) and was maintained during ischemia by another injection 1.5 h after the occlusion. Blood glucose levels were measured from tail vein blood using a glucometer (Freestyle, Alameda, CA).

Experimental temporary focal cerebral ischemia

Three h MCAO followed by 21 h reperfusion was used to induce transient ischemia. Isoflurane anesthesia was induced in an induction chamber and then animals were maintained for about 15 minutes on 3% isoflurane during surgery. The common carotid

artery was exposed through a midline cervical incision. The external carotid artery (ECA) was separated, ligated and cauterized. A heated rounded tip 4-0 nylon monofilament suture was advanced into the internal carotid artery to occlude the origin of the middle cerebral artery (MCA). Then, the monofilament was secured with one silk suture at the stump of ECA and the cervical incision was closed. After 3 h of ischemia the animals were re-anesthetized and the suture removed to allow reperfusion. Laser Doppler (Pim-3, Perimed, ST) was used to confirm a similar degree of drop in flow between groups. Core body temperature was monitored via a rectal probe and maintained using a heating pad under the animal during surgery and under the cage until the end of 24 h. Animals were singly housed before and after MCAO with free access to food and water.

Evaluation of neurovascular injury

Before sacrifice, all animals underwent intracardiac perfusion of ice-cold saline to flush out the blood from vessels. The brains were removed and the MCAs were isolated from ischemic and nonischemic hemispheres. Infarct size and presence of hemorrhage was analyzed in coronal slices of 2 mm thickness, labeled A-F, front to back. Visual inspection of hemorrhage was done, documented per slice if present and reported in a binary fashion as yes or no to indicate the frequency of macroscopic bleeding. 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., St. Louis, MS, USA) was used to outline the infarct area. Images were analyzed using specialized software recommended by NIH (Image-J). Image analysis was performed in a blinded fashion. The total infarct volume was reported as percent volume to the total ischemic hemisphere. Edema was expressed as a percentage of ischemic hemisphere size to control

hemisphere. The hemispheres were separated following staining and stored at -80^{o}C for later hemoglobin (Hb) direct ELISA analysis. HT was quantified by measuring excess Hb in the stroked hemisphere compared to the contra lateral side as published before [128].

Evaluation of MMP activity

For vascular MMP-9 activity, MCAs isolated from ischemic and nonischemic hemispheres were homogenized as reported previously [92]. Thirty µg homogenates or 20 µl (1:20 diluted) plasma were loaded directly on SDS-PAGE gel containing 0.1% gelatin and separated under non-reducing conditions. Following electrophoresis, the gel was washed twice in 2.5% "Triton X100" for 20 minutes each, rinsed with ddH_20 and incubated for 20 h in a substrate buffer- 50 mmol Tris-HCl, 5 mmol $CaCl_2$ + 0.02% NaN_3- pH= 7.5" at 37^{o}C. Recombinant MMP-9 active standard was run as positive control (Calbiochem, catalogue# PF024). Following incubation, the gel was stained using "Coomassie blue" for 3 h, then destained. The zymogram was digitized and band intensity was quantified by image analysis "GelPro analyzer, Media Cybernetics, MD" and expressed as the % of the recombinant standard [58, 129, 130].

Evaluation of neurological outcome

Ipsilateral circling, paw grasp and beam walk tests assessed function at baseline, before MCAO and at 24 h. Scores were graded 0-3 (normal-maximum deficit). Ipsilateral circling (no/few/several and continuous circling), paw grasp (normal grasp/ stroke side can touch/ stroke side hard to touch and unable to touch), beam walk (animal readily traverses/ walks slowly and shaking/ can stay or eventually falls and unable to stay for 10 seconds).

Neurological deficit was determined as a total score of these three tests, 9 indicating the worst outcome. In addition, Bederson's score was recorded at baseline and after occlusion to verify proper occlusion [131].

Statistics

The distributions for the measures of stroke severity (infarct size, percent edema and bleeding) as well as the measures of behavior were found to be skewed. A rank transformation was used prior to the analysis of these measures. The difference between blood glucose levels at different time points was determined by calculating the percent change from baseline at each time point which was then used in the within time-point analyses. The analysis for the effect of hyperglycemia on Wistar and GK rats was performed using a 2 disease (Wistar vs. GK) X 2 treatment (vehicle vs. hyperglycemia) ANOVA. An interaction between disease and treatment would indicate a differential effect of inducing hyperglycemia that is dependent on disease status. The analysis for the effects of hyperglycemia and metformin was performed using a 2 hyperglycemia (No vs. Yes) X 2 metformin (No vs. Yes) factorial ANOVA. An interaction between hyperglycemia and metformin would indicate a differential effect of metformin treatment dependent on hyperglycemic status. Tukey's test was used to adjust for multiple comparisons when determining mean differences for significant interactions.

Results

Physiological parameters

Baseline blood glucose values were significantly higher in diabetic than in control rats (Fig. 2.1). The aims of inducing HG with glucose injection were: 1) To achieve a range in control rats that is similar to the average blood glucose values in the diabetic GK rats (140-200 mg/dl) and 2) To overlay an acute increase in blood glucose in diabetic rats, both of which were successfully achieved. Metformin treatment achieved euglycemia in diabetic rats. There was no significant difference in arterial blood gases between control and diabetic rats (Table 2.1).

Blood glucose levels over the course of 24 h after anesthesia are depicted in (Fig. 2.1). After the initial glucose injection, Diabetic + HG group had significantly higher glucose levels than Diabetes + metformin +HG or Control + HG groups. During this experiment, there was a 2-step rise in blood glucose levels. The first rise was due to anesthesia that increased significantly in all groups. The second rise in glucose levels was due to MCAO that increased significantly from the anesthesia levels in three groups; Control, Diabetes, and Diabetes + metformin groups. The purpose of the second glucose IP injection midway during occlusion was to maintain HG during MCAO. At sacrifice, blood glucose dropped to similar levels in all groups.

Neurovascular damage

Infarct size was significantly lower in diabetic rats than in controls as we previously reported [58] (Fig. 2.2A). There was a disease and treatment interaction such that HG increased infarct size in diabetic but not in control rats. Metformin pretreatment did not affect infarct size in diabetic rats with or without additional acute HG (Fig. 2.2A). The percent reduction in blood flow from baseline after MCAO was similar in all groups indicating consistent occlusion (Fig. 2.2B).

Edema and hemorrhagic transformation (HT) were analyzed as indices of vascular damage. HT was assessed qualitatively by observing macroscopic hemorrhagic transformation and quantitatively by Hb ELISA (μg/g protein) (Fig. 2.3). Diabetic rats had a higher rate of HT than the controls (88% vs 13%, $p< 0.001$). Excess Hb and edema were higher in diabetes indicating vascular damage and there was a disease and treatment interaction such that HG increased both parameters in control but not diabetic rats groups (Fig. 2.3C and D). Pretreatment with metformin reduced brain edema in diabetic rats. While it did not affect the rate of HT, bleeding severity was reduced (Fig. 2.3C).

MMP activity

Since MMP-9 is associated with disruption of vascular integrity in ischemic brain injury, MMP-9 activity in both tissue (MCA) and plasma was measured using gelatin zymography (Fig. 2.4). MCA MMP-9 activity was greater in the nonischemic hemisphere of diabetic rats than in control rats indicating an upregulation with diabetes. Ischemia enhanced MCA MMP-9 activity in both control and diabetic rats. Metformin pre-

treatment did not affect MMP-9 activity on the nonischemic side but prevented the increase on the ischemic hemisphere. HG did not change MCA MMP-9 activity. Plasma MMP-9 activity at baseline was higher in diabetes. MCAO caused a dramatic increase as compared to baseline in all groups.

Neurological outcome

Short term (24 h) outcomes were assessed for all groups using a battery of neurobehavioral tests. Neurological deficits were more pronounced in diabetic rats and HG worsened the functional outcomes in both control and diabetic rats (Fig. 2.5).

Discussion

This study provides important evidence on the differential effects of acute hyperglycemia without diabetes versus diabetes that can be summarized as follows. A moderate acute elevation in blood glucose increases vascular damage (edema and HT) but does not worsen neuronal injury (infarct size) following stroke in otherwise healthy rats. Alternatively, overlaying acute hyperglycemia in diabetic rats exacerbates infarction but does not augment the HT or edema. Baseline MMP-9 activity is higher in the cerebrovasculature of diabetic rats. Ischemia/reperfusion injury causes a dramatic increase in MMP-9 activity with and without diabetes contributing to greater HT and edema in diabetes. Premorbid glycemic control improves vascular integrity and reduces edema in diabetes and acutely hyperglycemic diabetic rats. Most importantly, these results had an important impact on the functional outcome. The neurological deficit was greater in diabetic animals than in controls despite smaller infarcts. This is likely due to

increased brain edema and HT in diabetes. Worsened functional outcome in control animals with HG is associated with increased HT and edema. Altogether, these findings highlight the importance of vascular protection in acute hyperglycemic and diabetic ischemic brain injury.

The relative risk of ischemic stroke as well as short and long term unfavorable outcomes are significantly higher in patients with than without diabetes. Understanding the pathophysiology behind these outcomes may provide therapeutic alternatives for this high risk patient population [132]. Although it has been known that both diabetes and HG are predictors of poor ischemic stroke outcomes, limited knowledge exists about the different pathophysiologies, outcomes and the mechanisms of injury in each case [31, 32]. Clinical findings link admission HG, due to diabetes or not, to poor outcomes and to increased odds of HT. Alternatively, absence of diabetes and normoglycemia are both predictors of better clinical outcomes. The main goal of t-PA therapy is to achieve early reperfusion of the ischemic brain. However, when this happens in the presence of HG, the odds of HT increase which in turn limits the benefit of the only proven pharmacological intervention for acute ischemic stroke patients [34, 35]. Experimental studies on HG show larger infarct size and higher mortality rates after ischemic stroke with HG. Most of these studies employed severe hyperglycemia [52, 56, 59]. Few studies employed a true diabetes model and in most cases, diabetes was induced for a short period of time prior to MCAO rather than being a fully developed disease state [53]. We chose our model of diabetes since it has mild-moderate hyperglycemia (~ 180 mg/dl) more closely resembling the clinical situation where admission hyperglycemia is usually defined as blood glucose above 140 mg/dl [122]

[31] [37]. This is in contrast to most commonly used STZ models with severely elevated (~500 mg/dl) blood glucose. A second reason for selecting the GK model is that at the time of MCAO, the GK rats have been moderately diabetic for a considerably longer period of time, about 4 weeks.

The infarct size in our diabetic rats was smaller than control animals as we previously reported [58, 59, 129]. This may be due to diabetes-induced preconditioning which is known to be neuroprotective [133, 134]. Based on the results from GK we hypothesized that if there is preconditioning in GK rats, then glucose control would block this mechanism and the diabetic rats would develop infarct sizes similar to control rats. However, there was no significant change in infarct size due to metformin pretreatment. This would suggest that the smaller infarcts in the GK model are either not due to diabetes-mediated preconditioning or that this is a metformin specific protective effect. Recent studies reported that metformin is neuroprotective independent of its blood glucose lowering effect through its antioxidant and AMP-activated protein kinase (AMPK) stimulatory properties [135-137]. Future studies using different hypoglycemic agents are needed to address this point in more depth. At the levels of hyperglycemia that we achieved in the current study, ischemic damage in control HG rats was not different from control normoglycemic animals, yet when this level of HG was superimposed on diabetic rats, ischemic damage increased. These results point to a different neuronal injury pathophysiology in diabetes than in HG without diabetes and a possible association between the degree of hyperglycemia and neuronal injury.

Another important component of the neurovascular unit that has been studied less extensively than neurons is the blood vessels. Cerebral vasculature is a therapeutic

target and a good candidate for vascular protection strategies [79, 138]. Neurovascular unit integrity is compromised following prolonged periods of ischemia causing edema and HT due to disruption of the tight junction proteins and vessel breakdown. A common link between diabetes and ischemic stroke pathologies is MMP, which is upregulated in both diseases. MMP-9 is of special importance clinically and was found to predict higher HT risk. It is also postulated that the HT complicating t-PA therapy is mediated through upregulation of MMP-9 [68]. Admission blood glucose levels are a predictor of HT in patients given t-PA. Thus, controlling HG in ischemic stroke injury is likely to be important in maximizing both the therapeutic use and potential of thrombolytic therapy. When it is upregulated, MMP-9 activity degrades the basal lamina causing intracerebral bleeding. MMP-9 knockout mice were shown to be protected against ischemic stroke injury. In our study, 88% of diabetic rats exhibited intracerebral bleeding while this was 100% in HG animals versus 13% in control rats. Upon quantification, severity of HT was higher in diabetic rats compared to control. Edema was higher in diabetic and HG rats compared to control rats, suggesting that the cerebral vasculature is susceptible to hyperglycemia whether there is diabetes or not. It also points out that the threshold for vascular damage is relatively low and mild-moderate levels of HG are sufficient to cause a compromise in the vascular integrity of the neurovascular unit. Thus, controlling acute stroke HG and managing diabetes are of important in providing vascular protection against exacerbated ischemic brain injury. These findings provide further support for randomized clinical trials on the management of hyperglycemia in acute ischemic stroke [37, 119].

In the current study, we looked at MMP-9 activity in both cerebral macrovessels and in plasma to investigate the parallel patterns of activity in tissue versus circulation and to study the role of MMP-9 in HT in diabetes and HG. Baseline plasma MMP-9 activity was upregulated in diabetic compared to the control rats. Twenty-four h after MCAO, the MMP-9 plasma activity in all groups increased significantly up to the same level, due to the ischemic injury. A similar pattern was observed in cerebral macrovessels where type-2 diabetes upregulated MCA MMP-9 activity compared to control in the non-stroke side and ischemic injury caused a further elevation of MMP-9 activity in both control and diabetes macrovessels on the stroke side. The fact that bleeding frequency was significantly higher in both diabetes and HG rats than controls, yet the 24 h MMP-9 activity was not different between groups, indicates that earlier time points of plasma MMP-9 activity need to be investigated for proper correlation of plasma MMP-9 activity and HT for optimal utilization as an ischemic stroke biomarker [63].

Neurobehavioral outcomes are important tools for measuring the functional improvement or worsening after ischemic stroke. This study assessed neurological function at 24 h while the damage was still evolving. Nevertheless, the deficit was greater in the diabetic as compared to control rats and HG exacerbated the functional outcome in both control and diabetic rats suggesting that both acute and chronic hyperglycemia is critical for stroke outcome and may need to be evaluated independently. An interesting finding is that while premorbid glycemic control did not affect infarct size or bleeding, it improved neurological outcome. Pretreatment with metformin also reduced the deficit mediated by HG in diabetic rats. These findings strongly suggest diabetes and acute hyperglycemia need to be managed carefully to

improve stroke outcomes. However, more sensitive methods may better detect differences in neurobehavioral outcomes and that longer term outcomes may add further insight into the functional recovery of ischemic stroke in diabetes and in HG without diabetes.

Summary

Therapeutic options for ischemic stroke patients are limited. Given that 40-50% of acute ischemic stroke patients present with admission HG, understanding the different mechanisms of neurovascular injury in diabetes and HG, aids in giving a chance for better intervention and guideline improvement with a modifiable and manageable risk factor such as diabetes.

Table 2.1. **Arterial blood gases before and after MCAO**.
Before and after MCAO and postRe = post reperfusion.

	Control			**Diabetes**		
	before	after	postRe	before	after	postRe
pH	7.45±0.01	7.45±0.02	7.40±0.02	7.42±0.02	7.39±0.01	7.39±0.03
pCO2	41.37±0.83	40.78±2.10	49.12±2.54	48.68±2.46	51.61±2.60	47.91±2.88
pO2	163.83±1.54	168.50±3.52	120.20±11.05	150.18±4.08	161.00±4.47	133.43±9.87
N	6	4	5	11	8	7

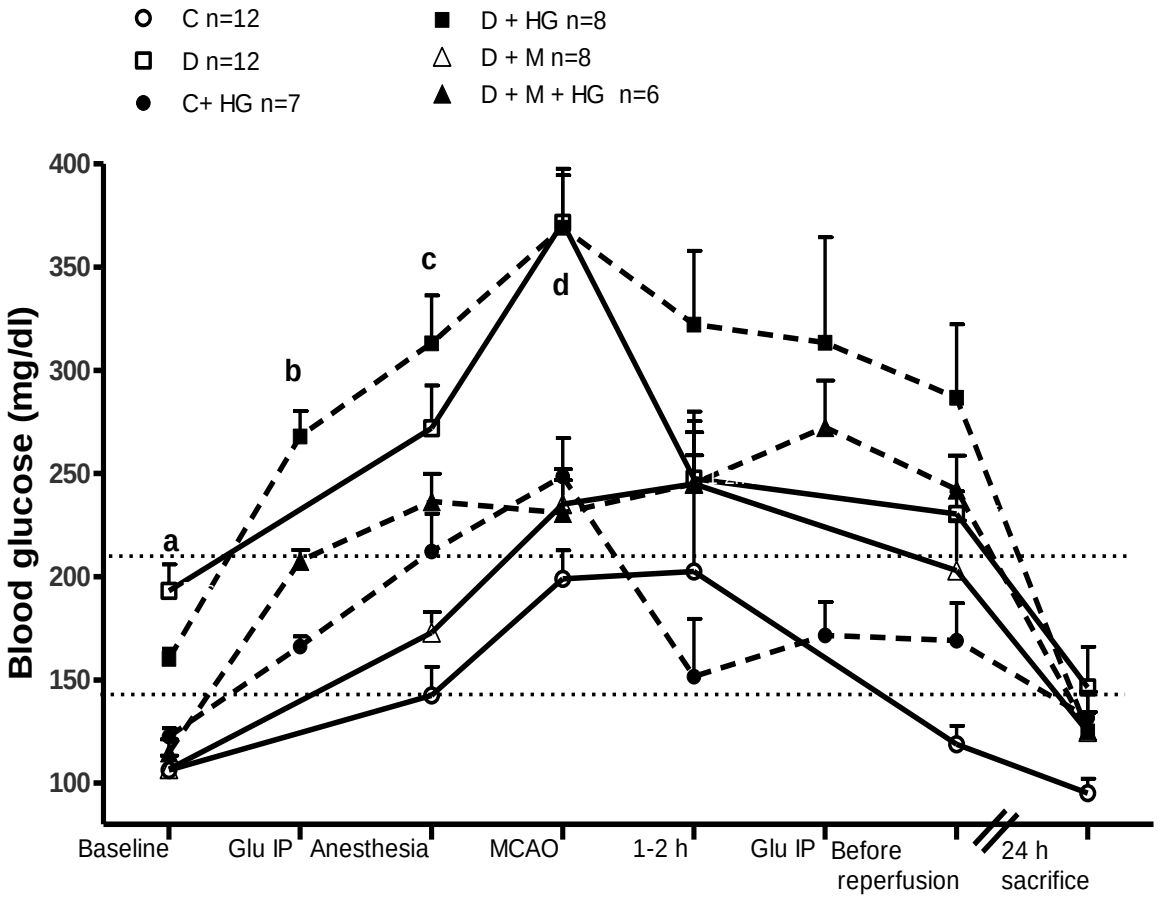

Fig. 2.1. **Blood glucose levels at baseline and over the course of MCAO in all study groups**. Glu IP= glucose intraperitoneal injection. Study groups were Control (C), Diabetes (D), C+ High Glucose (HG), D+HG, D+metformin (M) and D+M+HG. [a]$p<0.05$ D and D+HG vs other groups, [b]$p<0.001$ vs baseline, [c]$p<0.001$ vs Glu IP, [d]$p<0.005$ vs anesthesia in all groups with the exception of D+HG.

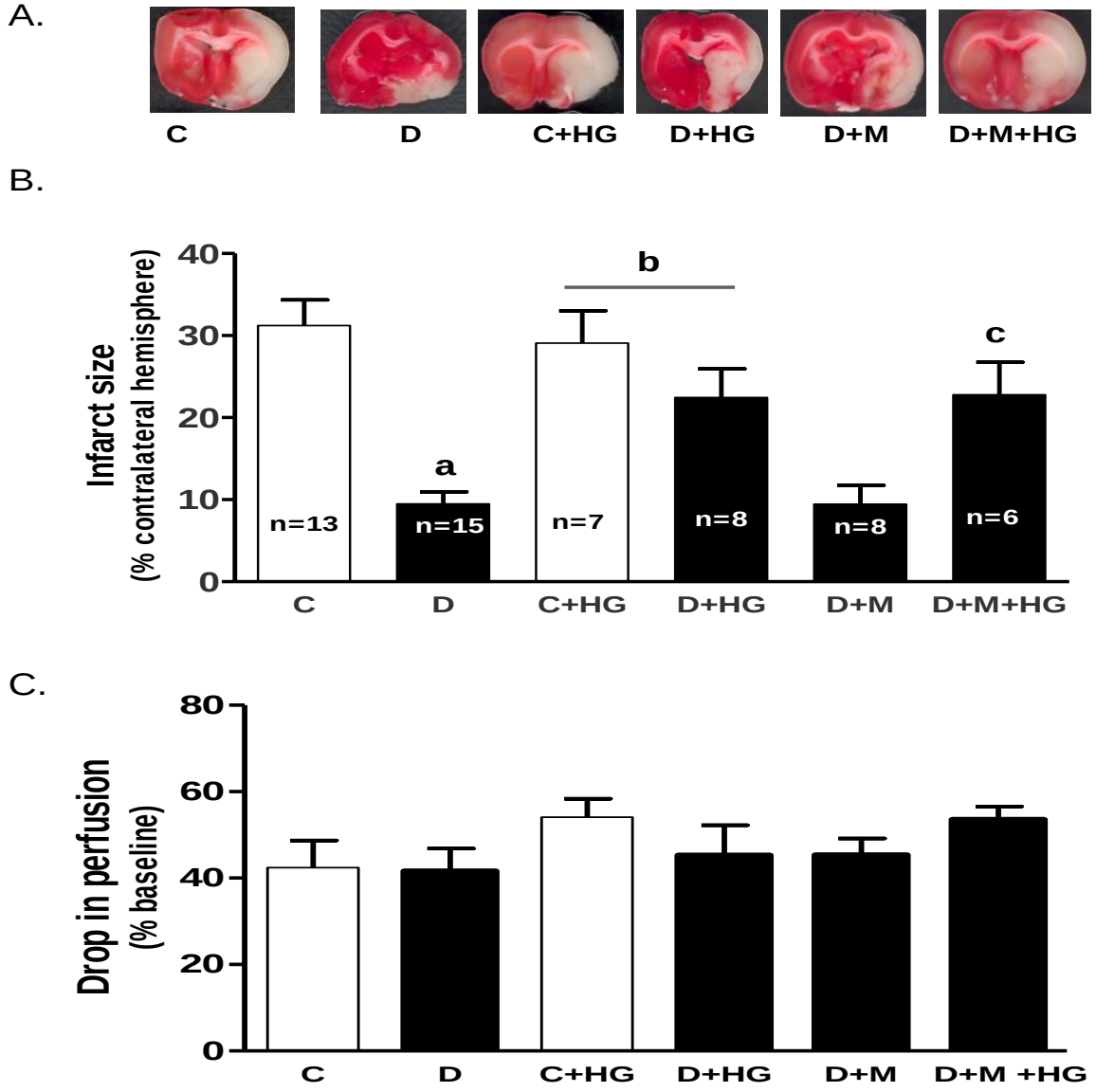

Fig. 2.2. **HG increases infarct size when superimposed on diabetes**. Representative images of ischemic damage determined by TTC stain and quantitative analysis of infarct size are shown in panels A and B, respectively. Drop in flow following MCAO (Panel C) was the same among groups. [a]$p < 0.05$ vs C, [b]$p=0.0062$ disease by treatment interaction compared to C and D, [c]$p=0.0035$ vs D or D + M.

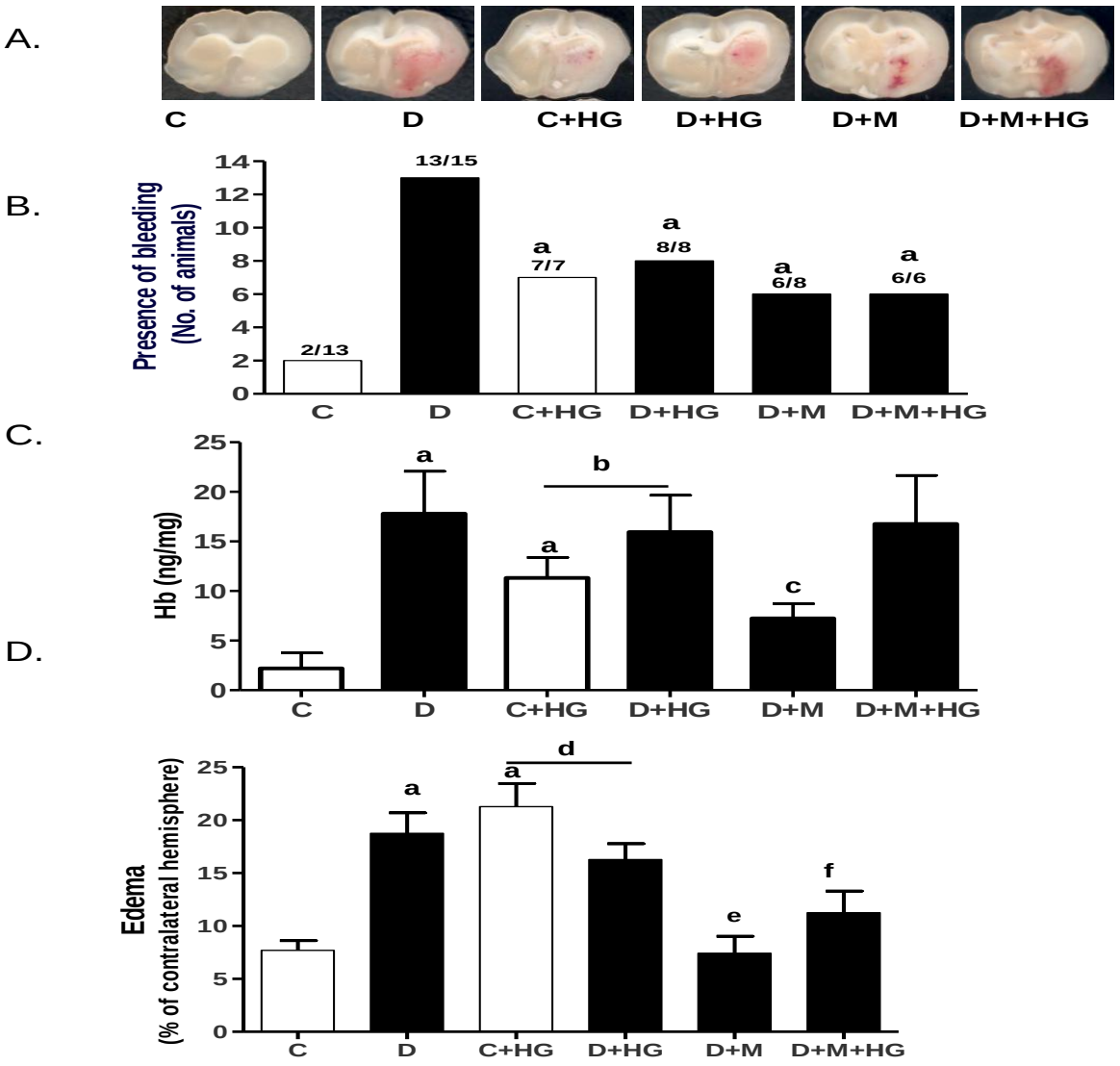

Fig. 2.3. **HG and diabetes augment vascular damage.** (A) Representative images showing hemorrhagic transformation (HT). (B) Frequency of macroscopic HT is significantly higher in diabetes and HG versus control. Severity of bleeding determined by excess Hb in the ischemic hemisphere (C) and edema (D) are greater in diabetes and HG. (D) . $^{a}p< 0.05$ vs C, $^{b}p=0.016$ disease by treatment interaction compared to C and D, $p< 0.001$ vs D, $^{c}p=0.0089$ vs D, $^{d}p<0.0001$ disease by treatment interaction compared to C and D, $^{e}p<0.0001$ vs D and $^{f}p<0.01$ vs D or D + HG.

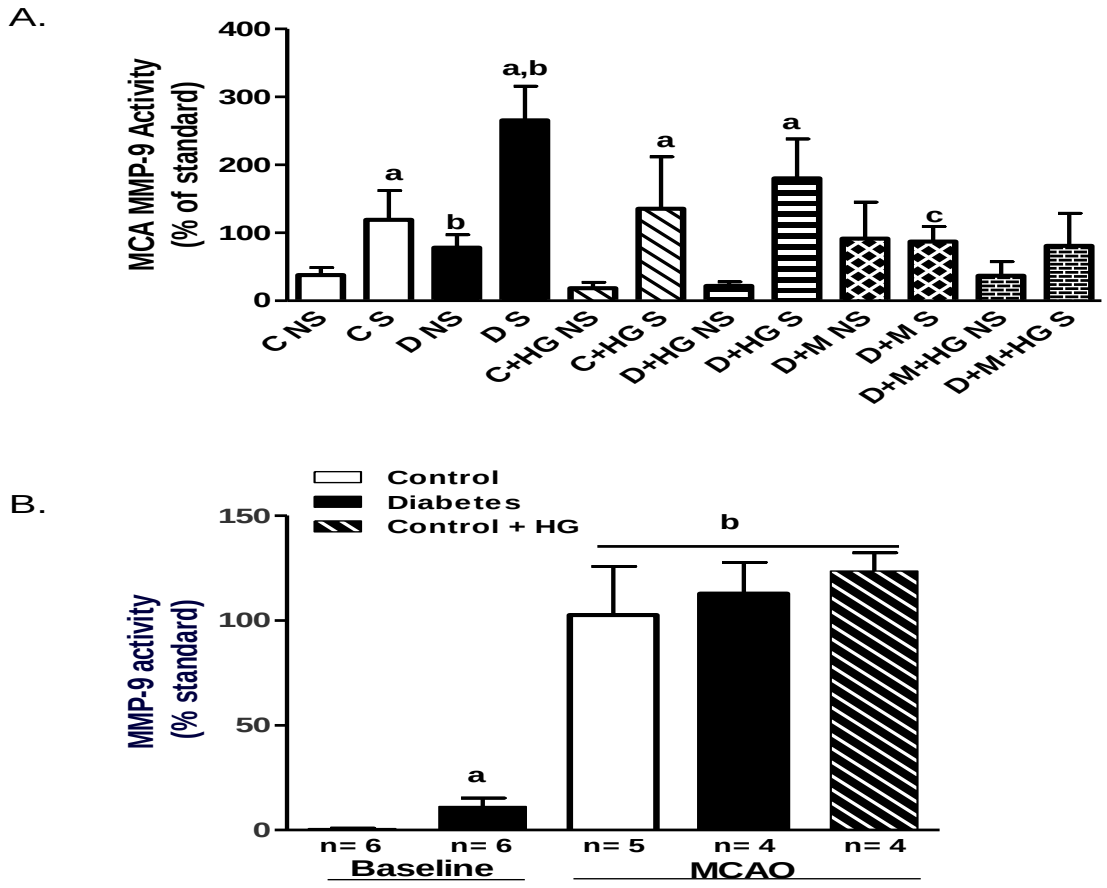

Fig. 2.4. **Local MCA and circulating MMP-9 activity under basal and ischemic conditions**. (A) MMP-9 lytic activity of the MCAs isolated from non-stroke (NS) and stroke (S) hemispheres as determined by gelatin zymography. Baseline (NS) activity was greater in diabetes than in control rats and further increased with ischemia. (B) Baseline plasma MMP-9 activity was higher in diabetes, and at 24 h after MCAO both diabetes and HG caused a significant increase. *p< 0.05 vs NS, **p< 0.001 vs C S, *** p< 0.001 vs D S.

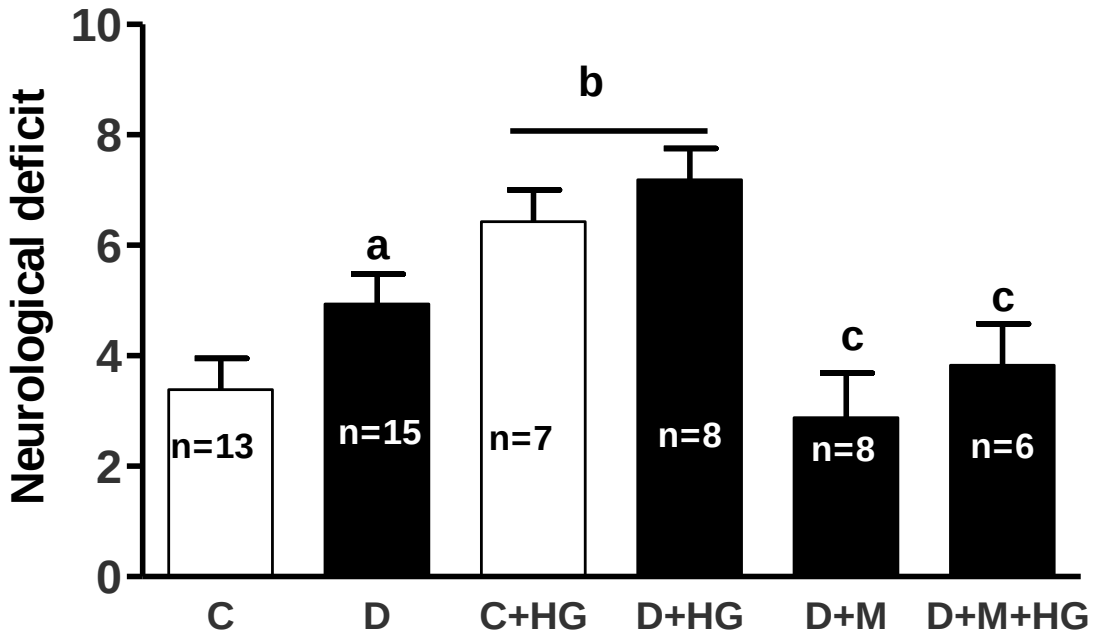

Fig. 2.5. **Effect of HG and diabetes on 24 h neurobehavioral outcomes.** Neurological deficit was determined as a composite score of ipsilateral circling, paw grasping, and beam walking tests (score of 9 indicating the worst outcome). [a]$p < 0.05$ vs C, [b]$p < 0.0001$ disease by treatment interaction compared to C and D, [c]$p < 0.001$ vs D or D + HG.

CHAPTER 3

VASCULAR PROTECTION IN DIABETIC STROKE: ROLE OF MATRIX METALLOPROTEASE-DEPENDENT VASCULAR REMODELING

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Abstract

Rationale: We have previously shown that middle cerebral artery occlusion (MCAO) causes greater hemorrhagic transformation (HT) in diabetic Goto-Kakizaki (GK) rats, a model with increased cerebrovascular matrix metalloprotease (MMP) activity and tortuosity.

Objective: To test the hypotheses that 1) Diabetes-induced cerebrovascular remodeling is MMP-dependant and 2) Prevention of vascular remodeling by glucose control or MMP inhibition reduces HT in diabetic stroke.

Methods and Results: Male Wistar control and GK rats were treated with vehicle, metformin or minocycline for 4 weeks. In one cohort, tortuosity index (TI), lumen diameter, number of collaterals between middle cerebral artery (MCA) and anterior cerebral artery (ACA) and the number of anastomoses within the MCA tree were measured as indices of remodeling. In a second cohort, MMP activity of MCAs was evaluated by zymography. A third cohort was subjected to 3 h MCAO/ 21 h reperfusion and infarct size and HT were evaluated as indices of neurovascular injury. All remodeling markers including MMP-9 activity were increased in diabetes and both metformin and minocycline prevented these changes. Infarct size was smaller in minocycline-treated animals and both metformin and minocycline reduced incidence and severity of HT.

Conclusion: These results provide evidence that diabetes-mediated stimulation of MMP-9 activity promotes cerebrovascular remodeling which contributes to greater HT in diabetes. Metformin and minocycline offer vascular protection that has important clinical implications for diabetes patients who are at a 4 to 6-fold higher risk for stroke.

Introduction

Diabetes is an increasingly growing epidemic affecting 21 million Americans over 65% of whom will eventually die of a macrovascular event such as stroke [1, 19, 84, 139, 140]. Since diabetic patients are at a higher risk of stroke and have poorer prognosis compared to the non-diabetic population, a better understanding of diabetes-induced vascular pathology and the underlying mechanisms is pivotal for developing better vascular protection strategies before and after an ischemic insult[132, 141, 142].

Traditional vascular complications of diabetes are categorized as: 1) Microvascular (nephropathy, neuropathy and retinopathy) and 2) Macrovascular (stroke, coronary artery disease and peripheral arterial disease) [11, 88, 89]. In both cases, the vascular wall structure and function are affected by remodeling changes. These may include vascular smooth muscle cells (VSMC) proliferation, degeneration of endothelial cells, basement membrane thickening and a state of coagulopathy [90]. In established disease, there is vascular wall growth as a result of increased collagen deposition and/or VSMC hypertrophy/hyperplasia [91, 143, 144]. The matrix metalloprotease (MMP) system is involved in restructuring of the vessels and the surrounding matrix by degrading as well as stimulating matrix deposition [145]. MMP-2 and -9 expression and activity are elevated under hyperglycemic conditions [100-102]. We have previously showed increased tortuosity and MMP activity of the cerebral vessels as evidence for early vascular structure changes in diabetes [58]. We recently reported that there is increased cerebral angiogenesis and arteriogenesis associated with augmented micro- and macrovessel MMP activity in diabetic animals [59]. We also

demonstrated that when diabetic animals are exposed to ischemic brain injury, they suffer greater hemorrhage and edema suggesting that preexisting adaptive neovascularization exacerbates stroke injury as also seen in diabetic retinopathy [58, 59]. Given that MMP-2 and MMP-9 rise acutely after stroke leading to edema and hemorrhagic transformation (HT) that develop secondary to prolonged ischemia [61, 65, 66, 146, 147], the relative contribution of diabetes-induced MMP activation and vascular remodeling to ischemic brain injury remained to be determined.

Blood flow is related to the fourth power of the vessel radius and thus even small changes in vessel caliber can have a significant impact on perfusion. Both structural and functional properties of cerebral vessels contribute to the regulation of lumen size. The cerebral vasculature has autoregulatory properties to adjust myogenic tone, a critical functional aspect of the cerebral circulation for adequate blood flow under normal conditions and more so in ischemia/reperfusion injury. Cipolla et al. reported that intrinsic myogenic tone of posterior cerebral arteries is diminished in response to increased glucose concentration *in vitro* [109]. However, Zimmermann et al. showed a constriction of MCAs in diabetic rats [110]. A third study reported that posterior cerebral artery tone is enhanced in diabetes [111]. Collectively, these studies emphasize the importance of understanding more about cerebral vessel structure and function under physiological circumstances and pathological alterations that may cause deleterious outcomes. As discussed above, we reported increased cerebrovascular remodeling and neovascularization in diabetes. Whether, and to what extent, these changes influence vascular tone, integrity and ultimately the magnitude of ischemia/reperfusion injury are yet to be determined. Taken together, our working hypothesis for the current study was

that MMP-mediated cerebrovascular remodeling in diabetes augments vascular damage following ischemia/reperfusion injury. We also hypothesized that tight glycemic control and/or MMP inhibition serve as vascular protection strategies.

Methods

Animals

The institutional care and use committee (IACUC) of the Medical College of Georgia approved all protocols used in the animal work. Male Wistar and Goto-Kakizaki (GK) rats were purchased from Harlan (Indianapolis, ID) and Taconic (Hudson, NY) Laboratories, respectively. For all studies, weight matched rats (270-310 g, 9-11 weeks) were used.

Metformin (MP Biomedicals, catalogue# 157805) was titrated to maintain euglycemia in GK rats (150-300 mg/kg/day based on blood glucose levels) and was given in drinking water artificially sweetened by non-caloric sweetener. Minocycline was also given in drinking water (5 mg/kg/ day, Sigma, catalogue# M9511). Both treatments were chronic starting with the onset of diabetes in GK rats till the animals reached the weight range used for MCAO which averaged about 5 weeks. Minocycline treatment was stopped 3 days prior to MCAO to allow for a wash-out period. Blood glucose levels were measured from tail vein blood using a glucometer (Freestyle, Alameda, CA).

Measurement of remodeling indices

Tortuosity index, collateral number and diameter were measured as indices of remodeling as described in detail in (Data supplement).

Evaluation of MCA vascular structure and myogenic tone

A segment proximal to the origin of MCA was isolated immediately following decapitation and mounted on the pressurized arteriography (Living Systems Instrumentation, Burlington, VT) to measure media thickness, lumen and outer diameters with a video dimension analyzer at different pressures ranging from 5-180 mmHg at 20 mmHg pressure increments. For details, please see data supplement.

Isolation of cerebral vessels

The animals were subjected to ischemic brain injury as described below. At 24 h, animals were sacrificed and macrovessels were isolated immediately from ischemic and nonischemic side separately, snap frozen in liquid nitrogen and kept at -80^{o}C for later protein work. Macrovessels are defined as basilar artery, MCA, circle of Willis and ACA. Vessels were homogenized using RIPA buffer to extract MMPs and a standard Bradford protein assay was done before running immunoblots or zymograms to determine the amount of loaded protein. In an additional group of animals treated with vehicle, metformin or minocycline, macrovessels were isolated at the end of the treatment period without any ischemic injury.

MMP-9 expression and activity

MMP-9 expression was determined by immunoblotting as described in detail (Data supplement).

Middle cerebral artery occlusion and cerebral perfusion measurement

Three h MCAO/21 h reperfusion model was used. Details of the procedure are given in the data supplement. Laser Doppler (PIM-3, Perimed, Stockholm, Sweden) was used to confirm a consistent drop in perfusion among groups. Core body temperature was maintained using a heating pad and monitored through a rectal probe during the surgery and on a heating pad under the cage until the end of 24 h. Animals were singly housed before and after MCAO with free access to food and water.

Evaluation of infarct size, edema and hemorrhagic transformation

At 24 h after occlusion, cerebral blood perfusion was evaluated with PIM-3 again and the animal was immediately sacrificed after perfusion with saline. Brains were enucleated and sliced in the coronal plane with 2 mm intervals, labeled A-F, front to back and were used to calculate infarct size, edema and HT. Please see the data supplement for details of evaluation.

Neurobehavioral assessment

Short term neurobehavioral functional outcomes of ischemic injury were assessed by a battery of tests including Bederson, fore paw grasp, beam walk, hind-limb retraction and elevated body swing test (EBST) tests at 24 h before sacrifice. The Bederson test was scored for the presence of forelimb flexion, decreased resistance to push and ipsilateral circling with each item given 1 point. A score of 3 is consistent with a middle cerebral artery occlusion. The Bederson's score was combined with the beam walking ability and bilateral forepaw grasp tests to determine a composite score [59]. Scores

were given to each item from 0 to 3 for a total of 9 for maximal deficit. The Bederson's score was also recorded at the end of 3 h occlusion period to verify proper occlusion [131].

Statistics

The distributions for the measures of stroke severity (infarct size, percent edema, and bleeding) as well as the measures of behavior and vascular remodeling were found to be skewed. The difference between stroke versus non-stroke side of the brain for MMP-2 and MMP-9 levels was calculated and the difference was adjusted for the non-stroke value in the analysis. A rank transformation was used prior to the analysis of all measures. The analysis for the effect of minocycline on Wistar and GK rats was performed using a 2 Disease (Wistar vs. GK) X 2 Treatment (vehicle vs. minocycline) ANOVA. An interaction between disease and treatment would indicate a differential effect of minocycline treatment that is dependent on disease status. The analysis for the effects of minocycline and metformin on GK rats was performed using a 3 Treatment (vehicle, metformin, minocycline) one-way ANOVA. Tukey's test was used to adjust for multiple comparisons when determining mean differences for significant ANOVA effects.

Results

Diabetes promotes vascular remodeling

Baseline physiological parameters are shown in table 3.1. Cerebrovascular structure was evaluated by two different methods: 1) Visualization and evaluation of cerebral pial vessels by PU4ii injection and 2) Pressurized arteriographic assessment of isolated MCAs. TI, collateral number, anastomoses number and collateral internal diameter were measured in PU4ii-injected animals as indices of remodeling of cerebral pial vessels. TI, as well as the number of collateral and anastomoses, were significantly higher in diabetic rats than in the control group. Both the metformin and minocycline treatments in diabetes reduced them significantly to control values (Fig. 3.1A-D). The collateral inner diameter (mm) was greater in diabetic GK rats and minocycline reduced this parameter significantly in both control and diabetic animals (Fig. 3.1E). Glycemic control with metformin also reduced inner diameter in GK rats.

Wall remodeling indices obtained from pressure arteriography included MCA inner and outer diameters (μm), cross sectional area (μm^2) and M/L ratio. In addition, myogenic tone and stiffness were assessed as a measure of vascular function and mechanics at an intraluminal pressure of 80 mmHg which represents the estimated pressure experienced by MCA in vivo. There was a disease and treatment effect on the inner ($p=0.0023$) and outer diameters ($p=0.01$) such that minocycline increased these parameters in controls but reduced them in diabetes. There was no difference in the cross sectional area or M/L ratio between groups (Fig. 3.2). Myogenic tone was significantly higher in diabetic rats than in controls at 80 mmHg pressure and both metformin and minocycline significantly

reduced the myogenic tone to less than control levels (Fig. 3.3A). There was a disease by treatment interaction effect on vascular stiffness. Minocycline reduced stiffness in controls but increased vascular stiffness in diabetes (Fig. 3.3B). Metformin had no effect on any of these parameters.

Diabetes augments stroke-induced increase in MMP expression and activity

MMP-9 is involved in the regulation of vascular remodeling and blood brain barrier breakdown following ischemic injury. Thus, MMP-9 activity was measured in macrovessel homogenates prepared from ischemic (I) and nonischemic (NI) hemispheres following MCAO. When NI hemispheres were compared as an indicator of baseline macrovascular MMP-9 activity, diabetic rats displayed greater enzyme activity than controls and glycemic control with metformin prevented this increase (Fig. 3.4A). Ischemic injury increased MMP-9 activity in both control and diabetic animals and metformin treatment prevented ischemia-induced MMP-9 activity in diabetic animals. Minocycline treatment abolished gelatinolytic activity in both control and diabetic animals. There was no difference in MMP-2 activity among the study groups or between ischemic and nonischemic macrovessels (Fig. 3.4B). In order to ensure that MCAO procedure itself is not affecting the MMP levels on the nonischemic side, macrovascular MMP expression/activity was determined at baseline without any exposure to ischemia in an additional group of animals treated with vehicle, metformin or minocycline. Both MMP-2 and MMP-9 activity were greater in the diabetic animals than in controls (Fig. 3.4C). Metformin treatment reduced MMP-2 but not MMP-9 activity in diabetic animals. Minocycline treatment lowered MMP-9 but not MMP-2 activity in both control and

diabetic rats. MMP-2 protein levels followed the same pattern with activity results (Fig. 3.4D). MMP-9 protein levels were higher in diabetic vessels but treatment with either metformin or minocycline did not have an effect.

Neurovascular injury is exacerbated in diabetes

The drop in perfusion after MCAO was consistent among groups (45-55 %, data not shown). Infarct size was significantly smaller in diabetic rats as reported by our group before (Fig. 3.5A and B). Treatment with metformin did not have an effect on infarct size but minocycline reduced infarct in both control and diabetic animals.

Edema and HT were evaluated as indices of ischemia-induced vascular damage. HT was assessed qualitatively by measuring the incidence of intracerebral bleeding and quantitatively by a previously validated hemoglobin ELISA. Diabetes caused greater vascular damage in diabetic rats than in controls as shown by the higher incidence of bleeding (Fig. 3.6A) and greater HT severity (Fig. 3.6B). There was a disease and treatment interaction such that minocycline significantly reduced HT incidence and severity in diabetes but not in control rats. Metformin treatment also reduced HT severity.

Neurological outcome is worsened in diabetes

Multiple neurobehavioral tests were used to assess the short term functional outcome of ischemia/reperfusion injury at 24 h. The composite neurodeficit score was significantly higher in diabetic animals indicating worse functional outcome (Fig. 3.7A). Treatment with metformin improved functional outcome. Minocycline treatment showed

a disease drug interaction with no effect on control animals but a reduced deficit in diabetic rats. There was no significant difference in EBST between groups (Fig. 3.7B). Minocyline improved fore paw grasp in both control and diabetic animals.

Discussion

The current study was designed to address the following important questions: 1) Is diabetic remodeling of the cerebrovasculature MMP-dependant?, 2) Does MMP-mediated cerebrovascular remodeling contribute to the augmented stroke injury seen in diabetes? and 3) Does glycemic control prevent MMP activation/remodeling and reduce neurovascular damage following ischemic brain injury? Our findings provide evidence that even after a short duration of relatively mild hyperglycemia, there are structural changes in the cerebral vessels as indicated by increased tortuosity, number of collaterals and collateral diameter all of which can be prevented by MMP inhibition. When these structural alterations are inhibited by minocycline treatment, vascular damage that accompanies ischemic brain injury is significantly reduced. Glycemic control prevents remodeling, reduces bleeding and improves functional outcome in diabetes.

Clinical data have shown remarkably worse outcomes, slower short and long term functional recovery and higher mortality in diabetic patients following stroke compared to the non-diabetic population [148-151]. Experimental studies mainly using streptozotocin (STZ)-induced model of diabetes with very high blood glucose levels also showed greater infarct development [42, 48, 53, 54, 120, 121]. Our understanding of the mechanisms involved in augmented ischemic injury in diabetes is limited. While much

emphasis is focused on neuronal damage following stroke, it is becoming clear that the vasculature plays an important role not only in the pathophysiology but also recovery of ischemic brain injury [59, 121]. We recently extended studies on diabetic stroke to a lean model of diabetes that presents with glucose levels (~200 mg/dl) that are comparable to levels seen in most acute ischemic stroke patients enrolled in various clinical trials [31, 37, 122]. Our studies have shown that the cerebrovasculature undergoes extensive remodeling leading to increased tortuosity and neovascularization that is associated with increased MMP activity in early diabetes [59]. We also reported that when these animals are subjected to temporary focal ischemia, the occurrence rate of overt hemorrhagic transformation increases significantly but infarcts are smaller [58, 129]. Numerous reports also documented the importance of MMPs especially during the acute phase of ischemia in damaging the neurovascular unit [70, 103, 147, 152-155]. The resulting loss of its crucial barrier function leads to edema and extravasations of red blood cells into brain parenchyma particularly with prolonged periods of ischemia. If the MMP system is dysregulated as occurs in diabetes, vascular wall integrity may be weakened setting the stage for an aggravated damage in case of stroke. The results of the current study show that chronic inhibition of MMPs by minocycline starting at the onset of diabetes prevents cerebrovascular remodeling and reduces HT incidence and severity. Since acute activation of MMPs during ischemia is important for brain injury, in the current study we stopped minocycline treatment 3 days prior to allow for a wash-out period to separate the effect of acute MMP inhibition on ischemic injury from that on vascular remodeling and neovascularization. Although there was no change in MMP-9 protein levels in diabetes with chronic minocycline treatment, enzyme activity on the

ischemic and nonischemic hemispheres were abolished compared to untreated diabetic rats. A possible explanation is that 3 day withdrawal is not sufficient to eliminate the inhibitory effect of minocycline on MMPs. Further studies are needed to clarify this issue.

Minocycline, although a non-specific MMPs inhibitor, was previously shown to inhibit cerebral MMP activity efficiently in experimental stroke [72, 155]. We also used it since it is a generic drug with a well known safety profile and because there is an ongoing clinical trial (MINO, Minocycline to Improve Neurologic Outcome) to evaluate its use as a neurovascular protective agent. In addition to its MMP inhibitory effects, minocycline has anti-inflammatory, anti-apoptotic and neuroprotective properties [156-159]. In the current study, minocycline-treated animals showed a small but significant decrease in infarct sizes as compared to vehicle treated control and diabetic animals. Thus, in the ischemia model used in this study minocycline was both neuro- and vasoprotective. It is possible that either the reduction of bleeding due to MMP inhibition decreases infarct size and/or direct neuroprotective effects contribute to this finding.

An important feature of the cerebral circulation is the ability to regulate blood flow within a wide pressure range to maintain the nutrient and oxygen supply to the brain. The mechanism behind this autoregulatory capacity is the myogenic reactivity of VSMC [108]. We wanted to study the effects of diabetes on this important functional feature of cerebral vessels. It is perceivable that structural wall alterations may affect the vascular wall function as well. This is especially true since previous studies showed different myogenic reactivity in different diabetes models [109-111]. In the current study, the myogenic reactivity across the pressure range (40-120 mmHg) was preserved in

diabetic animals. Although there was an increase in myogenic tone at 80 mmHg pressure, given that the lumen diameter is not different between control and diabetic rats at this pressure, it is unlikely to affect cerebral blood flow under normoxic conditions. However, it has to be recognized that we only measured myogenic reactivity and not neuronal or endocrine mechanisms that are involved in regulation of vessel diameter. In addition, whether vascular reactivity is altered under hypoxic conditions remains to be determined. Unexpectedly, both metformin and minocycline treatment reduced the tone significantly compared to vehicle treated animals which deserves further investigation.

The chronic hyperglycemia present in diabetes ultimately leads to both micro- and macrovascular remodeling changes. One of the goals behind glycemic control in diabetes is to prevent both these changes and hence prevent and/or reduce diabetes-dependant vascular events. It is well established that early and good glycemic control reduces the microvascular complications in both types of diabetes. However, the relation between glycemic control and macrovascular events prevention has been only proven in type-1 diabetes [82]. The most recent major prospective randomized controlled clinical trial ACCORD (Action to Control Cardiovascular Risk in Diabetes) was prematurely stopped due to the increased macrovascular events leading to mortality in the tight control arm [26, 82-87]. However, ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation) and VADT (Veteran Affairs Diabetes Trial) did not replicate the same findings [21-23]. The latest consensus statement by the American Diabetes Association highlights that despite the non-significant reduction in macrovascular events in these 3 trials, the long term follow-up of

DCCT and UKPDS showed that early control with target HbA_{1C} around 7% is associated with long term reduction in macrovascular events and supports the overall benefits of glycemic control in reducing vascular events in addition to the importance of controlling other comorbidities [26]. Previous work from our group showed evidence of cerebrovascular remodeling in diabetes [58]. We also showed that glycemic control prevents microvascular remodeling in the mesenteric bed [99]. Yet, whether diabetes-dependant cerebrovascular remodeling contributed to augmented ischemia/reperfusion injury remained unclear. The cerebrovascular remodeling was indeed inhibited and vascular damage was reduced via glycemic control (metformin treatment). Minocycline was used to inhibit MMP activity which decreased both cerebrovascular remodeling and HT. Collectively, these data support our hypothesis that diabetes induces MMP-9 upregulation which in turn leads to higher neurovascular injury after ischemia/reperfusion injury.

Our results provide evidence that diabetes-induced MMP-9 activity upregulation promotes cerebrovascular remodeling and affects vascular myogenic reactivity as well. This remodeling is associated with higher vascular damage following ischemia/reperfusion injury which may explain in part at least why diabetic patients have a worsened injury compared to the non-diabetic population. Glycemic control is important to reduce vascular damage associated with ischemic brain injury. The clinical application of minocycline ability to reduce both incidence and severity of HT makes it a vascular protective agent. This is a safe and feasible strategy that can be used to benefit patients with diabetes who are at higher odds of ischemia/reperfusion injury.

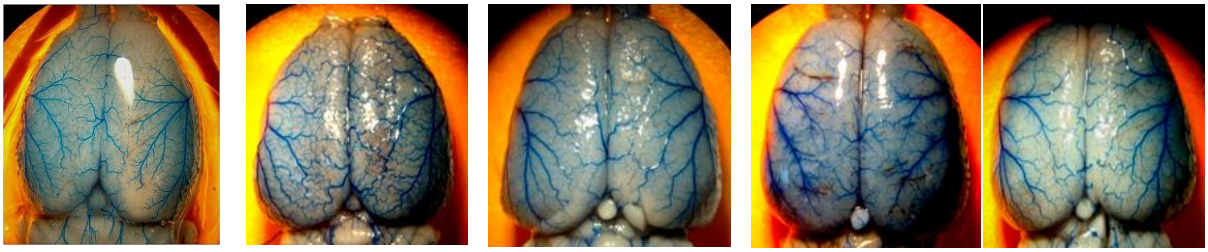
Acknowledgments

The authors thank Kamakshi Sachidanandam for technical assistance with arteriography. This work was supported by grants from NIH (DK074385, NS054688), American Heart Association Established Investigator Award (0740002N) to A.E. and Department of Veterans Affairs Merit Awards to S.C.F. and A.E.

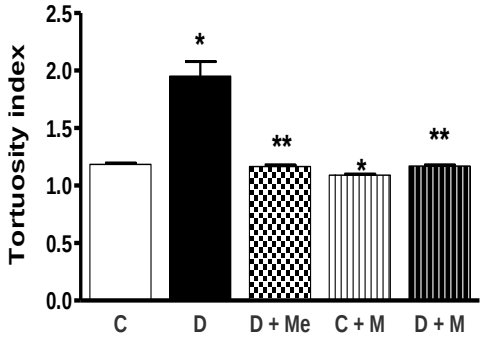
Table 3.1. **Baseline metabolic parameters**. *p< 0.001 vs Diabetes.
BW = body weight and BG = blood glucose.

	Control	Diabetes	Diabetes + Metformin	Control + Minocycline	Diabetes + Minocycline
BW (g)	293.6 ± 9	279.2 ± 10	256.0 ± 4	316.3 ± 4	270.9 ± 3
BG (mg/dL)	110.0 ± 11*	212.0 ± 19	106.8 ± 7*	102.2 ± 8*	177.4 ± 21

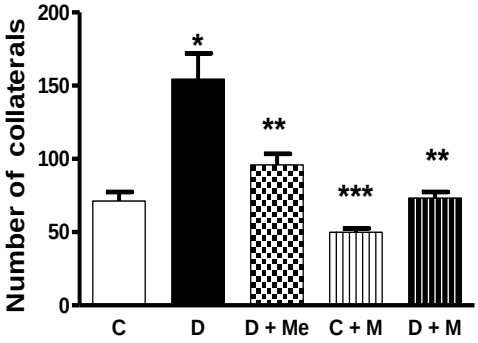
A.


C
D
D + Me
C + M
D + M

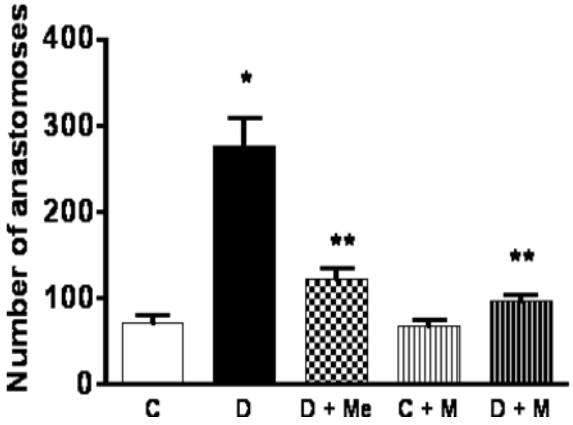
B.


Tortuosity index
2.5
2.0
1.5
1.0
0.5
0.0
*
**
*
**
C
D
D + Me
C + M
D + M

C.


Number of collaterals
200
150
100
50
0
*
**
***
**
C
D
D + Me
C + M
D + M

D.


Number of anastomoses
400
300
200
100
0
*
**
**
C
D
D + Me
C + M
D + M

E.

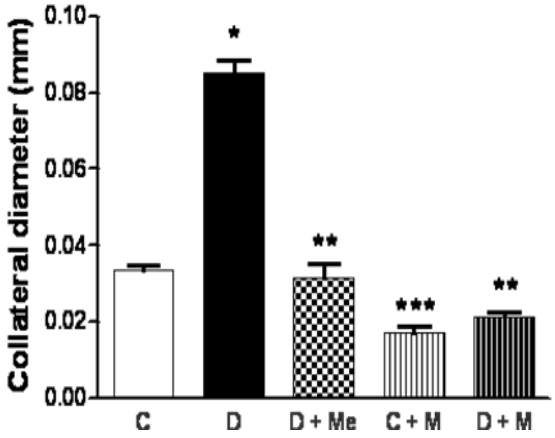

Collateral diameter (mm)
0.10
0.08
0.06
0.04
0.02
0.00
*
**
***
**
C
D
D + Me
C + M
D + M

Fig. 3.1. **Diabetes promotes remodeling of cerebral vessels**. (A) Representative images of the pial vessels after PU4ii injection to visualize MCA, ACA and PCA trees in control (C), diabetes (D), diabetes + metformin (D +Me), control + minocycline (C + M) or diabetes + minocycline (D + M) groups. TI (B), number of collaterals (C), number of anastomoses (D) and collateral diameter (E) were significantly higher in diabetes all of which were prevented by metformin or minocycline treatments. Mean ± SEM, n=7-14, *p<0.0001 vs C, **p<0.001 vs D, ***p<0.0002 vs C.

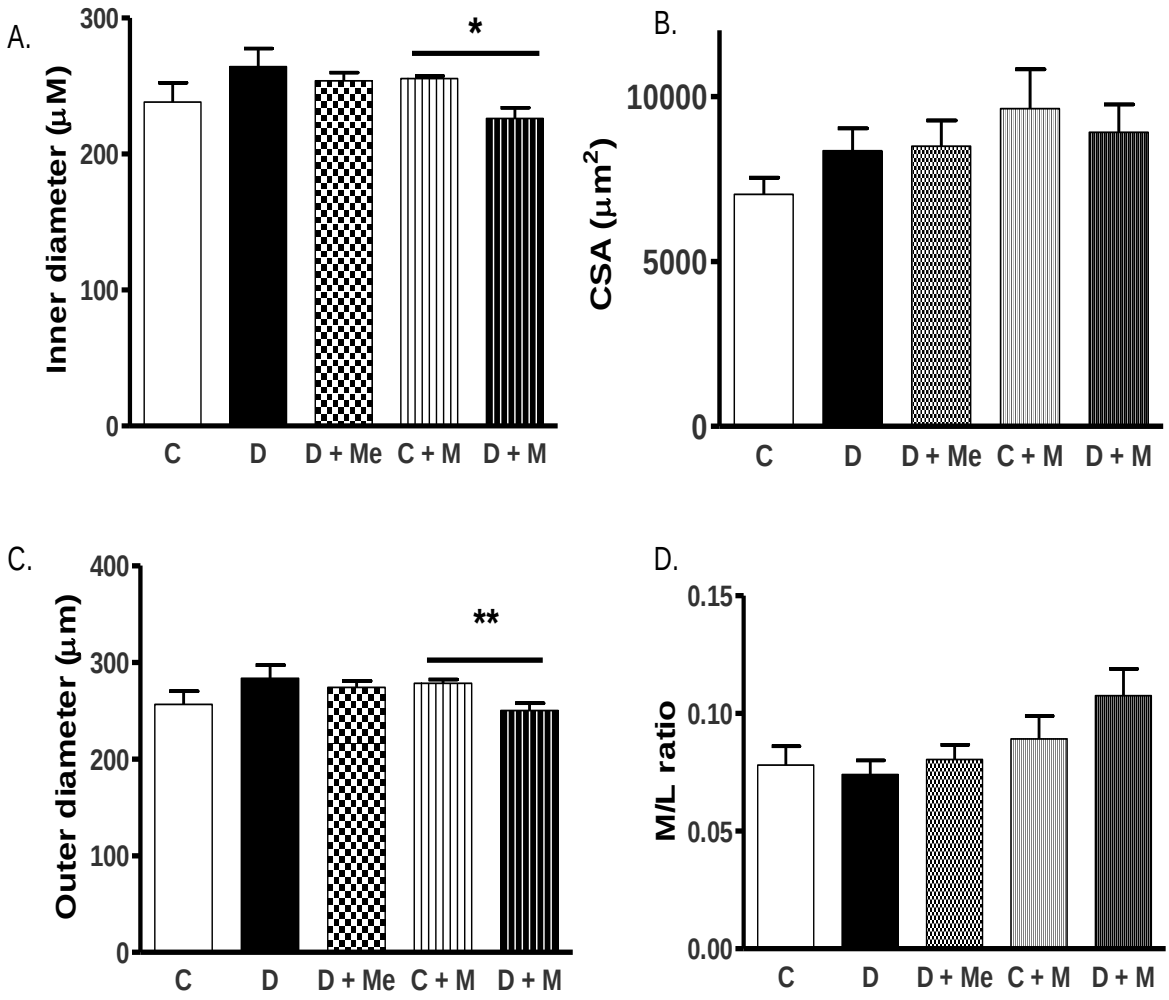


Fig. 3.2. **Despite the remodeling changes see in pial vessels, distal MCA vessel wall did not show increased wall thickening or M/L ratio in diabetes**. MCA wall remodeling parameters including (A) inner diameter, (B) cross sectional area (CSA), (C) outer diameter and (D) M/L ratio were measured by pressure-arteriography at 80 mmHg. Mean ± SEM, n=6-9, *p=0.0023 and **p=0.01, Disease by treatment interaction.

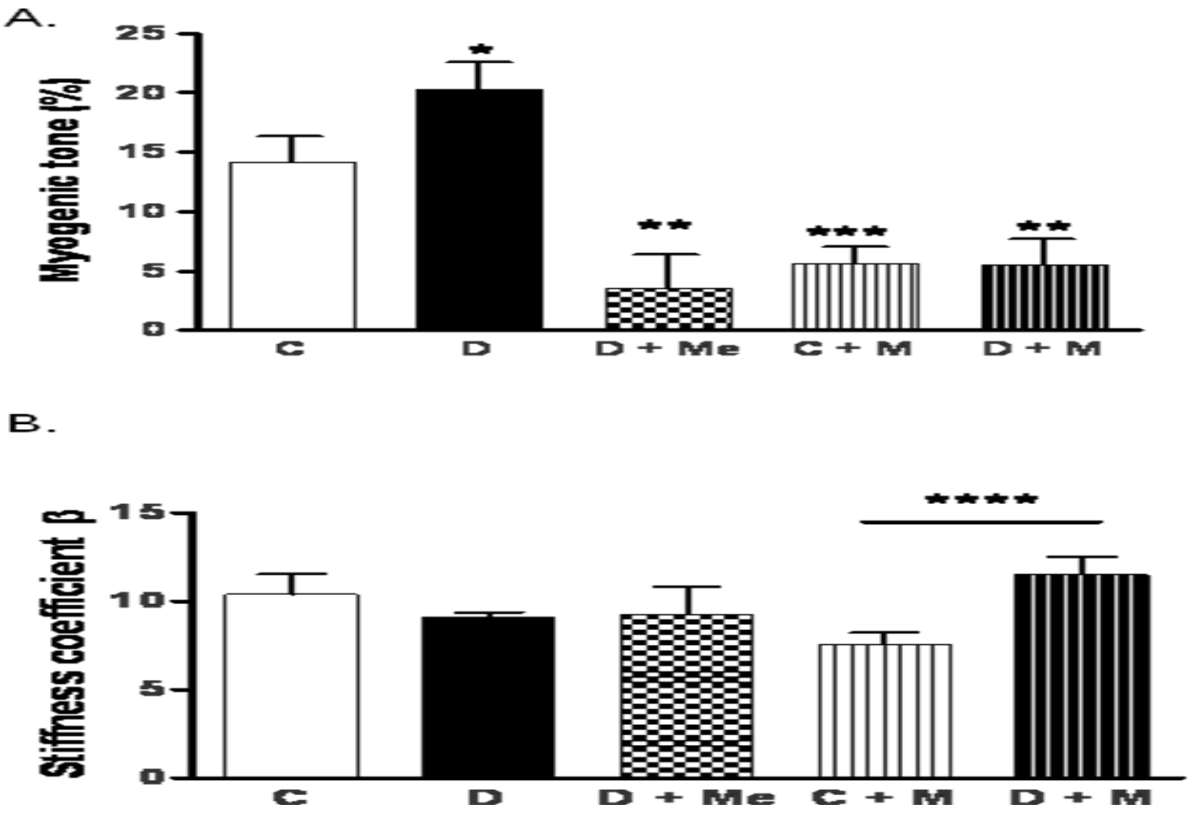


Fig. 3.3. **Diabetes increases myogenic tone of isolated MCAs**. (A) MCA myogenic tone was increased in diabetic animals and both metformin and minocycline reduce the tone. (B) There was a disease and treatment interaction such that minocycline decreased stiffness in controls but increased it in diabetic rats. Mean ± SEM, n=7-14, *p<0.05 vs C, **p=0.011 vs D, ***p=0.0046 vs C.

.

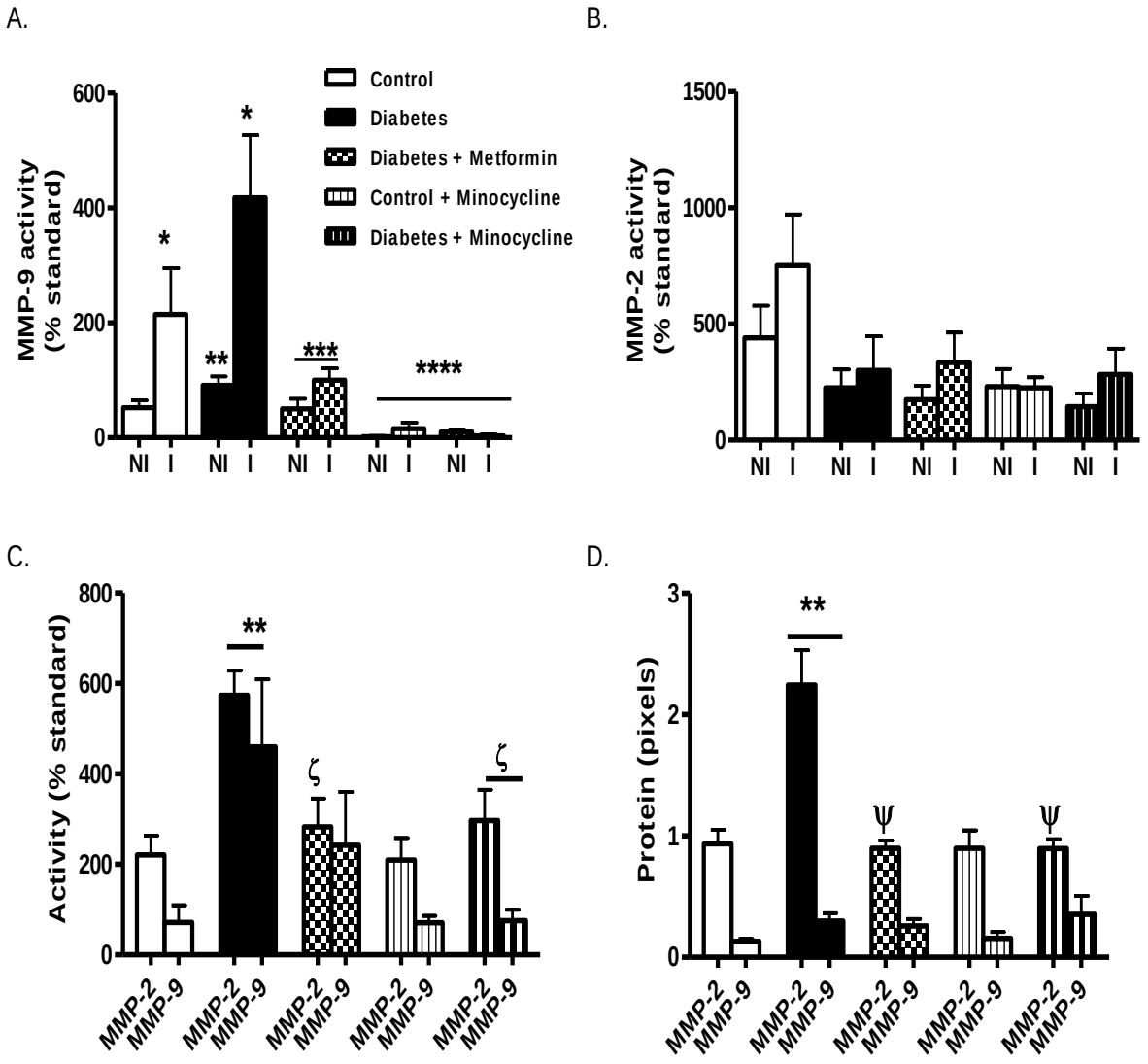


Fig. 3.4. **Diabetes augments ischemia-induced stimulation of MMP-9 activity in isolated cerebral vessels**. (A) MMP-9 activity in macrovessels isolated from ischemic (I) and nonischemic (NI) hemispheres of animals subjected to MCAO was measured using gelatin zymography. MMP-9 activity was greater in both the NI and I hemispheres in diabetes indicating increased baseline and ischemia-induced augmentation of MMP-9

activity. Both metformin and minocycline cause a dramatic reduction in enzyme activity. (B) MMP-2 activity of the same groups did not show any difference. (C) When lytic activity was assessed in the same cerebral macrovessels isolated from animals not subjected to MCAO, both MMP-2 and MMP-9 activities were greater in diabetes. (D) Protein levels of MMP-2 and MMP-9 were increased in diabetes. Metformin and minocycline treatments significantly reduced MMP-2 level but not MMP-9. Mean ± SEM, n=5-8, *p<0.01 vs NI, **p=0.05 vs C, ***p=0.012 vs D, ****p<0.0001 vs vehicle C or D, $^{\varsigma}$p=0.031 vs D, $^{\Psi}$p=0.003 vs D.

A.

B.

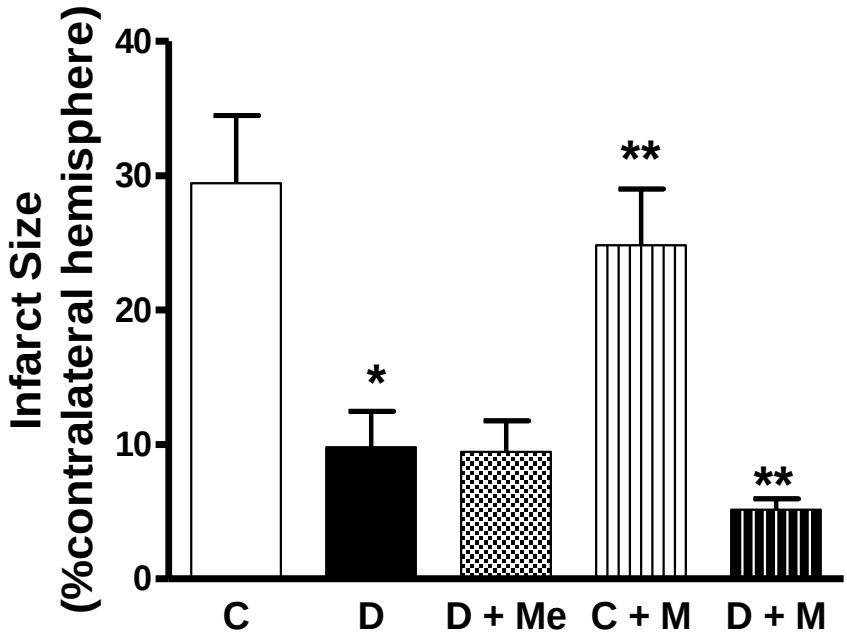


Fig. 3.5. **Infarct size is smaller in diabetes**. (A) Representative images of TTC-stained coronal sections of the brain following MCAO. (B) Quantitative analysis of infarct size indicated smaller infarcts in diabetes. While metformin had no effect on infarct size minocycline reduced infarct in both groups. Mean ± SEM, n=6-9, *p<0.0001 vs C, **p=0.05 vs vehicle C or D.

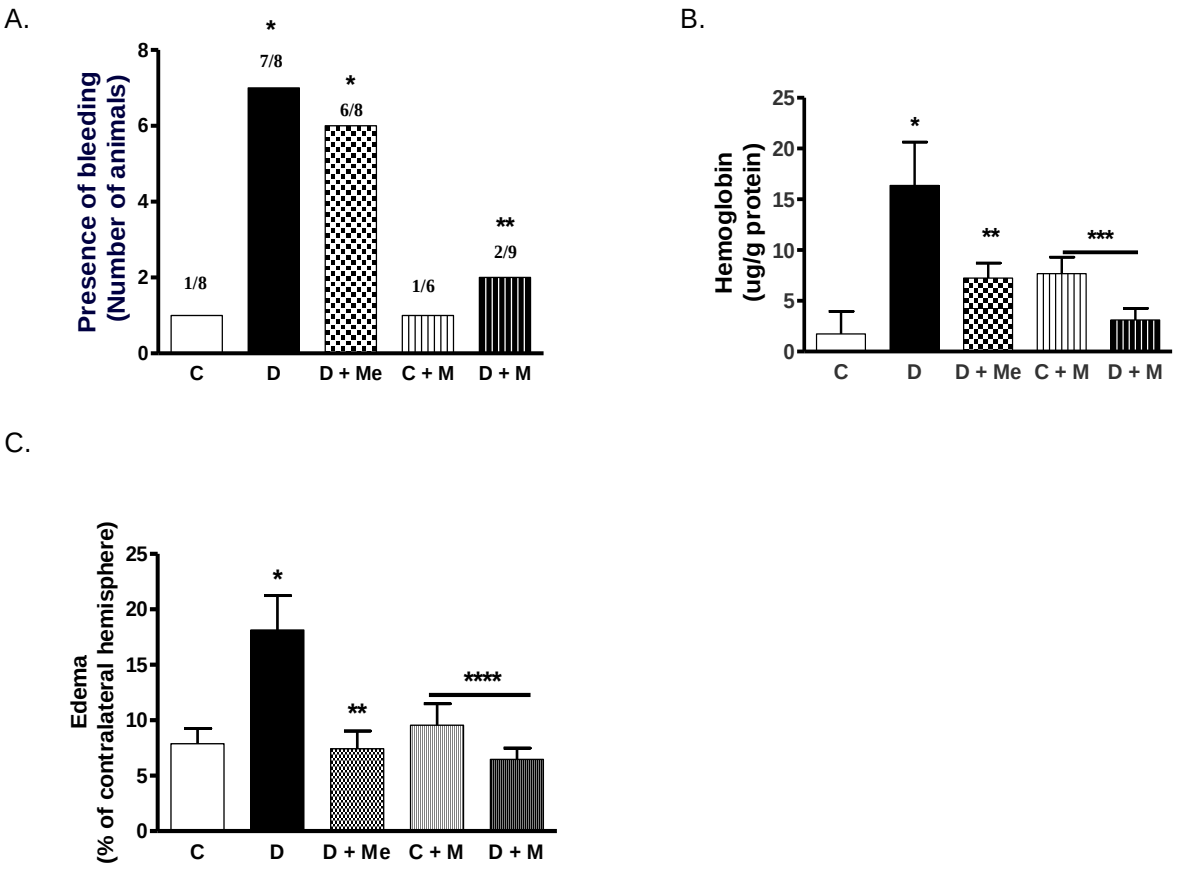


Fig. 3.6. **Vascular injury is greater in diabetes**. (A) Occurrence of macroscopic intracerebral bleeding was significantly higher in diabetes compared to control and was reduced by chronic minocycline treatment. (B) ELISA measurements of hemoglobin show significant HT in diabetes and a vasoprotective effect of minocycline and metformin. There was a disease and treatment interaction showing minocycline preventing HT in diabetes but no effect on control animals. C) Edema was significantly higher in diabetes compared to control and both metformin and minocycline reduced it. Minocycline had no effect on edema in control animals indicating a disease/treatment interaction. Mean ± SEM, n=6-11, *p<0.05 vs C, **p=0.001 vs D, ***p=0.0009, ***p=0.0024 disease/treatment interaction for minocycline.

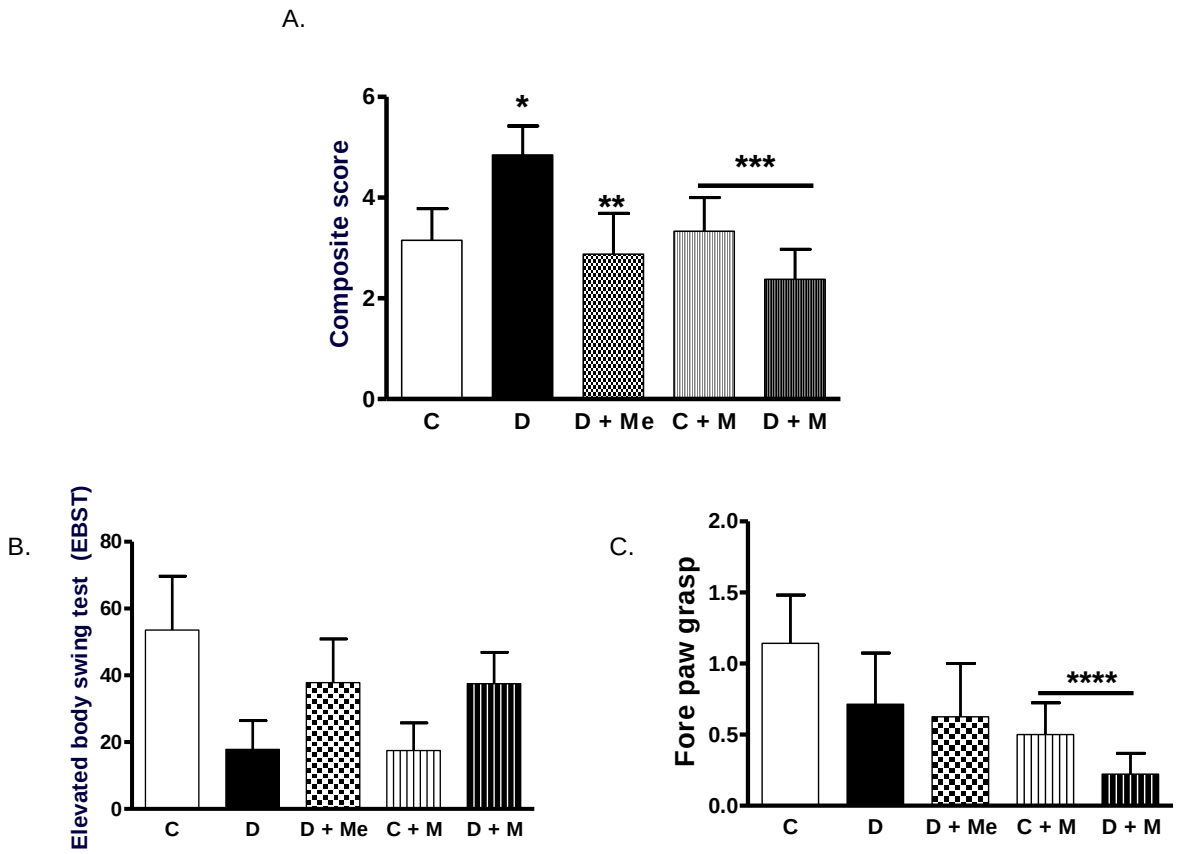


Fig. 3.7. **Short-term (24 h) neurological outcomes following MCAO in all treatment groups.** A) A composite score for multiple neurobehavioral tests shows worse functional recovery in diabetes compared to control. Both metformin and minocycline treatment improved the score significantly. Minocycline showed a differential effect indicated by no change in control animals but improvement of score in diabetic animals. While there was no difference in individual scores of EBST (B) and paw grasp (C) was improved in minocycline treated animals. Mean ± SEM, n=6-13, *p<0.05 vs C, **p=0.05 vs D, ***p=0.035 disease/treatment interaction for minocycline, ****p=0.0031 vs vehicle C or D.

Data supplement

Measurement of remodeling indices

After being anesthetized with sodium pentobarbital (100 mg/kg), the animals were injected with 3 ml freshly prepared polyurethane elastomer PU4ii (vasQtec, Switzerland) through the aorta within a minute. PU4ii mixture was prepared by mixing the blue-stained ethylmethylketone (30% of the final mixture) and 0.8 g of PU4ii hardener shortly before casting as previously described [160]. The rats were decapitated immediately and the brain was removed and immersed in 4% paraformaldehyde for 48 h.

Stereomicroscopic images of the perfused brains were captured and vessel length of cortical branches of MCA starting from its origin was traced on a Wacom tablet 493-3 using Image J I-36 software. Each hemisphere was divided into 6 grids of equal area and the total number of collaterals between the anterior (ACAs), posterior (PCAs), and middle cerebral arteries (MCAs) were counted manually [161]. A collateral was defined as an anastomosis between MCA and ACA or PCA. Arteriole-to-arteriole anastomoses between the MCA branches were also counted and defined as intratree anastomosis. The diameter was measured at the midpoint of the collaterals. At least 8 measurements of the diameters were taken per hemisphere and the average was reported as the diameter of the collaterals. The tortuosity index (TI) was defined as the ratio of the vessel length over straight line distance between two vessel ends. In each grid, 2 middle size vessels were traced and the average of 12 measurements was used as the TI.

Evaluation of MCA vascular structure and myogenic tone

One of the MCAs collected immediately following decapitation was mounted on the pressurized arteriography (Living Systems Instrumentation, Burlington, VT), to measure media thickness, lumen and outer diameters with a video dimension analyzer at different pressures ranging from 5-180 mmHg at 20 mmHg pressure increments. The system was equilibrated in Krebs-HEPES buffer free of calcium to obtain measurements under passive conditions and media thickness, lumen and outer diameters and vessel cross-sectional areas were determined as follows: Wall Thickness (WT, μm) = Outer diameter - lumen diameter (i.e., OD - LD); (M/L) ratio = WT/LD; Cross Sectional Area (μm^2) = Outer vessel area - lumen area. Myogenic tone and stiffness of MCAs (β-coefficient) were calculated as follows: Myogenic Tone (% tone) = (1 - ($OD_{active}/OD_{passive}$)) x 100; Stiffness (β-coefficient) was obtained from the slope of the stress vs strain curve using the equation: $y = ae^{\beta x}$ (Y= stress, X= strain, a= intercept, β= slope) [162]. The other MCA was cannulated and pressure fixed at 80 mmHg for 30 minutes in 10% neutrally buffered formalin solution, sectioned and stained with Masson trichrome for morphometry.

Isolation of cerebral vessels

The animals were subjected to ischemic brain injury as described below. At 24 h, animals were sacrificed and macrovessels were isolated immediately from ischemic and nonischemic side separately, snap frozen in liquid nitrogen and kept at -80^{o}C for later protein work. Macrovessels are defined as basilar artery, MCA, circle of Willis and ACA. Vessels were homogenized using RIPA buffer to extract MMPs and a standard Bradford

protein assay was done before running immunoblots or zymograms to determine the amount of loaded protein. In an additional group of animals treated with vehicle, metformin or minocycline, macrovessels were isolated at the end of the treatment period without any ischemic injury.

MMP-9 expression and activity

MMP-9 expression was determined by immunoblotting. Fifty µg proteins were directly loaded on SDS-PAGE gel and separated under reducing conditions. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Five % milk-TTBS was used for blocking and band detection was done using primary antibody against MMP-9 (Calbiochem, catalogue# IM37L) and a peroxidase conjugated goat antimouse secondary antibody. The chemiluminescent signal was detected using (Alpha Imager) and bands intensity quantified by image analysis using GelPro analyzer (Media Cybernetics, MD).

MMP-9 activity was assessed by gelatin zymography. Protein was loaded in 30 µg on SDS-PAGE gel containing 0.1% gelatin and separated under non-reducing conditions. After electrophoresis, the gel was washed twice in 2.5% “Triton X100” for 20 minutes twice and incubated for 20 h in a substrate buffer- 50 mmol Tris-HCl, 5 mmol $CaCl_2$ + 0.02% NaN_3- pH= 7.5 at 37^oC. A recombinant MMP-9 standard was run as positive control (Calbiochem, catalogue# PF024). Following incubation, the gel was stained using “Coomassie blue” overnight then destained. The zymogram was digitized and bands intensity was quantified by image analysis GelPro.

Middle cerebral artery occlusion and cerebral perfusion measurement

Three h MCAO/21 h reperfusion model was used. Animals were anesthetized using isoflurane in an induction chamber then maintained for about 15 minutes on 3% isoflurane for the procedure. A midline cervical incision was made to expose the common carotid artery. The external carotid artery (ECA) separated, ligated and severed. A rounded-tip, by heating, 4-0 nylon monofilament suture was inserted into internal carotid artery to occlude the origin of MCA [163]. The occlusion suture was secured with one silk suture at the stump of ECA and the incision was closed. After 3 h the animals were re-anesthetized and occlusion suture removed to allow reperfusion. Laser Doppler (PIM-3, Perimed, Stockholm, Sweden) was used to confirm a consistent drop in perfusion among groups. Core body temperature was maintained using a heating pad and monitored through a rectal probe during the surgery and on a heating pad under the cage till the end of 24 h. Animals were singly housed before and after MCAO with free access to food and water.

Evaluation of infarct size, edema and hemorrhagic transformation

At 24 h after occlusion, cerebral blood perfusion was evaluated with PIM-3 again and the animal was immediately sacrificed. Brains were enucleated and sliced in the coronal plane with 2 mm intervals, labeled A-F, front to back and were used to calculate infarct size, edema and HT. Visual inspection of hemorrhage if present was reported as a qualitative score for the frequency of macroscopic bleeding. 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., St. Louis, MS, USA) was used to outline the infarct area. Images analysis was performed in a blinded fashion using the Image-J (NIH, MD) software. Following staining, hemispheres were separated; snap frozen at -80^{o}C for later

hemoglobin direct ELISA quantification [128]. Edema was expressed as a percentage of infarct hemisphere size to control hemisphere.

Neurobehavioral assessment

Short term neurobehavioral functional outcomes of ischemic injury were assessed by a battery of tests including Bederson, fore paw grasp, beam walk, hind-limb retraction and elevated body swing test (EBST) tests at 24 h before sacrifice. The Bederson test was scored for the presence of forelimb flexion, decreased resistance to push and ipsilateral circling with each item given 1 point. A score of 3 is consistent with a middle cerebral artery occlusion. The Bederson's score was combined with beam walking ability, and bilateral forepaw grasp tests to determine a composite score [59]. Scores were given to each item from 0 to 3 for a total of 9 for maximal deficit. Bederson's score was also recorded at the end of 3 h occlusion period to verify proper occlusion [131].

CHAPTER 4

DISCUSSION

AIS patients face multiple challenges clinically that limit the use and benefits of the only FDA-approved drug. Since the inception of the NINDS t-PA trial, early thrombolysis to restore reperfusion with t-PA has become the gold standard in practice. The aim is to salvage the penumbra by restoring the flow of oxygen and nutrients, reduce infarct size and improve functional outcomes. Unfortunately, in reality only 5% or even less of the targeted patient population actually receive the therapy. Limiting factors include:

1. Time window to reach the hospital, conclusively diagnose ischemic stroke and exclude a hemorrhagic on in order to administer t-PA is limited, although it was recently extended to 4.5 h [164].
2. The major adverse side effect of t-PA itself which is HT.
3. Successful recanalization rates of 50%.

The unsuccessful clinical quest for finding a newer neuroprotective agent led to the STAIR (Stroke Therapy Academic Industry Roundtable) committee recommendations [165]. In essence, they aim at a future successful transition of promising drugs from the bench to human clinical trials. This is following the failure of many experimental drugs to show a clinical benefit so far. In addition, the search among many molecules for a potential AIS biomarker is an active area of research that is still developing and showing promise. Collectively, there is an urgent need for newer

therapies for AIS patients, newer therapeutic approaches that can improve the current outcomes of prognosis and or maximize the therapeutic potential of t-PA and a surrogate biomarker that correlates well with AIS outcomes. The current study was designed to investigate the above aspects of these clinically relevant questions in AIS injury in type-2 diabetes.

Traditionally, the BBB has been regarded as the anatomical boundaries of specialized endothelial cells between the CNS and peripheral circulation for the protection of the former from harmful external environment stimuli. Nowadays, the definition has evolved into a more integrated functional aspect of the neurovascular unit [166-168]. Within the neurovascular unit, the microvascular tissue and neurons communicate actively to regulate the function of each other with the facilitation of astrocytic connections while wrapped inside an intact extracellular matrix. The impervious barrier function is maintained all the time by tight junction proteins and basal lamina. The I/R injury is a double injury affecting more than one target tissue, infarct (neuronal injury) and HT (vascular damage) [169]. The neuronal damage has gained most of the focus in the search for novel neuroprotective agents. However, searching for strategies that will maintain vascular integrity is another approach for neurovascular protection that has been less pursued so far [79, 138]. Vascular damage takes place secondary to prolonged ischemia following the occlusive event. If reperfusion is not restored, the vessels will collapse, bleeding occurs and AIS is said to have undergone conversion “HT”. In diseases where vascular complications are well documented such as type-2 diabetes, the integrity of vessels is more detrimentally affected compared to healthy subjects. This pre-existing vasculopathy may explain, in part the worse

outcomes observed clinically in situations where AIS and HG coexist. There is little known about the molecular mechanisms behind such augmented injury in diabetic AIS patients. Also, the distinction between the injury patterns and outcomes in hyperglycemia versus type-2 diabetes is not well defined.

The clinical relevance of these questions is highlighted by the fact that admission hyperglycemia is present in 30-50% of AIS patients and not all of them are diabetic [121]. If the injury and outcomes are different, that will have an impact on the proper care measures to be taken in each set of these patients. Since hyperglycemia and type-2 diabetes are therapeutically manageable risk factors in AIS patients as shown by recent major clinical trials, this approach provides a valuable tool in improving the current guidelines as well as the outcomes for diabetic AIS patients.

Three major clinical trials that recently concluded were able to show the safety and feasibility of controlling hyperglycemia in AIS patients. In the THIS and the GRASP trials, majority of patients were diabetic while in the GIST-UK they were hyperglycemic patients [36, 37, 170]. The 3 month survival analysis in either trial did not show a difference between the control and treatment groups since more subjects were needed. Future studies that are adequately powered to answer questions regarding glycemic control efficacy are under way or pending. The results of ACCORD, ADVANCE and VADT were controversial regarding the efficacy of intensive glycemic control in improving type-2 diabetes-induced macrovascular complications. However, in a recent expert consensus report by the American Diabetes Association, the American College of Cardiology and the American Heart Association different explanations were listed in order to clear the controversy [26]. In summary, explanations are:

1. Design flaws related to unambiguously meaningful clinical outcomes.
2. Unbalanced control of CVD comorbidities between the control and treatment arms ultimately leading to skewed results.
3. Control was in a population where HA_{1C} was low to begin with so no further benefits were shown and no extrapolation to patients who were poorly controlled (higher HA_{1C}) should be done [26].
4. The stage of type-2 diabetes in participants was advanced and a follow up study in younger participants showed CVD benefits from glycemic control [171].
5. A recent UKPDS follow up study points out that maybe glycemic control is of greater benefit before the macrovascular events are well established [13].
6. The specific glycemic control strategies used, whether pharmacological or non-pharmacological, seem to have an effect on the outcome and current trials are designed to answer this question more specifically [171].

Previous data by our group showed smaller infarct size yet higher vascular damage (bigger edema and higher incidence of HT) in our rodent model of type-2 diabetes, GK rats [58]. The results were confirmed by a different study in STZ-induced model of diabetes, so they were not model specific (unpublished data). We also demonstrated vascular remodeling where different remodeling indices, TI and MMPs activity, were significantly higher in type-2 diabetes animals compared to the control. Based on these results, we formulated our hypothesis that type-2 diabetes promotes vascular remodeling via MMPs dysregulation and the resulting vascular pathology leads to worse outcomes in AIS. We also hypothesized that controlling hyperglycemia and or inhibiting MMPs should ameliorate AIS outcomes.

The first aim of the study was designed to study neurovascular damage and short term neurobehavioral outcomes in type-2 diabetes versus hyperglycemia. We originally postulated that acute hyperglycemia would increase the degree of neuronal damage. This was true only in type-2 diabetes when blood glucose levels were further increased and infarct size significantly increased. However, when the same mild levels of acute hyperglycemia were achieved in control animals, we observed the same degree of neuronal injury as in control. Alternatively, glycemic control with metformin did not change the infarct size in type-2 diabetes. Both type-2 diabetes and hyperglycemia resulted in vascular damage that was manifest as more edema and higher HT. Chronic pretreatment with metformin showed vascular protection via significantly decreasing the type-2 diabetes-induced vascular damage. The short term neurobehavioral outcome was worse in the case of hyperglycemic compared to type-2 diabetes.

One of the appealing biomarkers for AIS is MMP-9. This is because clinical data show an association between its activity and HT. As mentioned earlier, the hemorrhagic conversion due to prolonged ischemia and or t-PA therapy itself is an unfavorable outcome that specifically happens more frequently following early reperfusion with t-PA when patients suffer from admission hyperglycemia. Consequently we hypothesized that baseline plasma and/or cerebrovasculature MMP-9 would be higher in type-2 diabetes. Our results confirmed the hypothesis. Baseline plasma MMP-9 activity, before stroke, was higher in type-2 diabetes animals compared to controls. Twenty-four h after AIS plasma MMP-9 activity was further elevated to a similar threshold level following the I/R injury in control, type-2 diabetes and hyperglycemic animals. In cerebral macrovascular tissue, baseline MMP-9 activity and expression were significantly higher

in type-2 diabetes compared to control and 24 h after AIS, type-2 diabetes + HG upregulated MMP-9 activity in the stroke side compared to the non-stroke hemisphere.

The second aim of the study investigated the impact of type-2 diabetes-induced cerebrovascular remodeling on AIS injury and the efficacy of glycemic control and MMPs inhibition on mitigating I/R outcomes. We examined the structure of cerebral vessels using different approaches. The PU4ii dye injection provided us with morphological information about the pial cerebrovascular trees and the intertwining anastomoses. The pressure arteriography preserved the vascular patency and shape to study remodeling, tone and mechanics under conditions as close as possible to the *in vivo* environment. Histochemical staining for sections in MCA was also performed to study the morphometry of cerebral vessels. There were many significant type-2 diabetes-induced cerebrovascular remodeling changes. TI was higher as shown by us before. The number of collateral vessels, anastomoses and inner diameter of collaterals were all higher in type-2 diabetes compared to control. However, wall remodeling parameters from pressure arteriography showed no significant changes in inner diameter, outer diameter or cross sectional area. The remodeling index M/L ratio was not significantly different between type-2 diabetes and control animals. Quantitative analysis of Masson trichrome stained sections of MCA confirmed these results. Both glycemic control with metformin and MMPs inhibition with minocycline chronic treatments were able to prevent the type-2 diabetes-induced changes in TI, collateral vessels number, anastomoses number and collaterals inner diameter. At baseline, type-2 diabetes led to an upregulation in both the expression and activity of MMP-9 in cerebral macrovessels. In microvessels the baseline MMP-9 expression levels were

also higher in type-2 diabetes animals but activity was undetectable. In neuronal tissue MMP-9 levels were not different between type-2 diabetes and control.

Minocycline chronic treatment was successfully able to inhibit MMP-9 activity to baseline but metformin treatment was not. Following AIS, neither metformin nor minocycline treatments reduced the neuronal injury or edema. Minocycline treatment was vascular protective in type-2 diabetes where both the incidence and severity of HT were significantly reduced.

CHAPTER 5

SUMMARY AND CONCLUSIONS

In a rodent type-2 diabetes model, neuronal injury is augmented by further acute elevation in blood glucose levels and infarct size gets bigger. The mild levels of acute hyperglycemia used in the study were not sufficient to increase the infarct size suggesting a higher level of hyperglycemia is needed. The smaller infarct size seen in type-2 diabetes suggests that chronic exposure to hyperglycemia i.e. the duration might trigger some neuroprotective mechanisms.

Cerebral vessels became however more susceptible to I/R damage augmented by hyperglycemia independent of type-2 diabetes. HT increased significantly compared to control following mild acute elevation in blood glucose in type-2 diabetes and acute hyperglycemia animals. Early chronic treatment with metformin to regulate hyperglycemia immediately following type-2 diabetes onset provided vascular protection by decreasing HT severity and edema size. These results highlight the importance of early and sustained glycemic control in type-2 diabetes as a useful and feasible vascular protective strategy in AIS.

Type-2 diabetes caused upregulation of baseline plasma and cerebral macrovascular MMP-9 activity and this elevation is further increased by: 1) Additional

acute elevations in blood glucose values and 2) I/R injury. Since MMP-9 is linked to HT, these results point to the importance of glycemic control in type-2 diabetes following AIS to avoid further vascular damage. In microvessels despite the fact that MMP-9 expression was higher in type-2 diabetes, no detectable activity was observed perhaps due a differential posttranslational regulation.

Twenty-four h macrovascular MMP-9 activity was not different between treatment groups in the stroke side compared to the contra-lateral non-stroke side. Studies investigating earlier time point are needed to elucidate the time points of MMP-9 activity upregulation.

Type-2 diabetes indeed induced ongoing cerebrovascular remodeling process starting early on with the disease onset which we postulated would cause a preexisting vascular pathology explaining in part the worsened AIS injury outcomes seen in type-2 diabetes. Metformin and minocycline were both able to prevent these early type-2 diabetes-induced remodeling changes. However, the observed remodeling was not fully developed at this stage to affect wall parameters or M/L ratio significantly. These conclusions are supported by our study results in older animals where the M/L ratio of cerebral vessels was significantly higher in type-2 diabetes older animals compared to the younger ones. In addition chronic treatment with metformin was beneficial in reducing the M/L in the older type-2 diabetes animals. These data show that even after type-2 diabetes induces significant wall remodeling changes, they still can be reversible with tight glycemic control.

Type-2 diabetes-induced remodeling is a dynamic process which is affected by the disease stage. The data indicate that cerebral pial vessels morphology is affected first followed by wall parameters later on in macrovessels. Whether or not microvessels are affected was not part of the study design and warrants further studies. The fact that early control of hyperglycemia and or inhibition of MMPs prevent this remodeling gives clues to possible new therapeutic intervention strategies. Even after the wall remodeling progressed, metformin treatment was still able to reduce it and decrease the M/L ratio in older type-2 diabetes animals.

The original postulation was that the type-2 diabetes-induced MMP-9 upregulation was responsible for the remodeling and thus could be a mechanistic link behind the higher HT in type-2 diabetes and that metformin treatment to control hyperglycemia and/or minocycline treatment to inhibit MMP-9 should be able to ameliorate AIS injury in type-2 diabetes. In the case of minocycline, this was true since it provided vascular protection and improved short term neurobehavioral outcomes in type-2 diabetes following AIS.

CHAPTER 6

LIMITATIONS

Although HT was evident in type-2 diabetes and the baseline MMP-9 expression and activity were upregulated, 24 h activity of MMP-9 correlated more with I/R injury in all groups. It seems that at 24 h the MMP-9 activity has already reached a peak following I/R injury. Thus, further studies assessing the temporal profile of earlier time points are warranted for a sound conclusion to associate MMP-9 activity with HT and AIS injury. Also, the same studies are warranted in the previously investigated tissues namely plasma, neurons and cerebral microvascular and macrovascular tissues.

There could a technical issue related to the results obtained about myogenic tone and mechanics from pressure arteriography. Although all experiments for all treatment groups were done under the same conditions, the intramural pressure that was used to aid the vessels develop tone was lower than what is usually reported. It is estimated that *in vivo* MCAs experience a pressure of 80 mmHg and 10 mmHg was used. Whether or not the tone and mechanics data were affected by that, warrants further investigation. However the structural and remodeling data obtained from pressure arteriography were confirmed by 2 other methods.

Since there is no specific MMP-9 inhibitor so far, we decided to use an inhibitor, minocycline, with a well established safety profile, that has been shown to effectively inhibit MMP-9 and that is currently used in our institution in an ongoing NIH-funded clinical trial as a novel neuroprotective agent. Minocycline is also anti-inflammatory so parts of the observed effects can be related to that mode of action as well. Despite the 3 days wash-out period to ensure passage of at least 5 $t_{1/2}$ periods, we still observed unexpected neuroprotective effects of minocycline.

The fact that metformin did not reduce baseline MMP-9 activity could be related to the specificity of the antidiabetic agent used and not the fact that glycemic control has no effect on reducing MMP-9 activity. Further studies using different hypoglycemic agents would shed a better light on this point.

The significance of metformin's ability to reduce M/L in older animals on AIS injury outcomes in hindered by the general technical difficulty in stoke field to apply the MCAO model on bigger animals.

Another issue related to using young animals that fit within a weight range suitable for MCAO technique is that they are healthy and have not had enough time to allow disease states progression contrary to the clinical AIS population. However in our type-2 diabetes model, animals have been diabetic for 4 weeks which is relatively a much longer period compared to most studies on hyperglycemia in stroke where it was induced for a brief period of time usually for few days prior to MCAO.

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