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Cerebral microvascular matrix metalloproteinase-3 (MMP3) contributes to vascular injury after stroke in female diabetic rats

Yasir Abdul[1,2], **Sarah Jamil**[1,2], **Weiguo Li**[1,2], **Adviye Ergul**[1,2]

[1]Department of Pathology & Laboratory Medicine, Medical University of South Carolina,

[2]Ralph H. Johnson Veterans Affairs Health Care System, Charleston, SC

Abstract

Diabetes exacerbates hemorrhagic transformation (HT) after stroke and worsens clinical outcomes. Female patients with diabetes are at a greater risk of stroke and worsened recovery. We have shown that activation of matrix metalloprotease 3 (MMP3) in hyperglycemic settings mediates HT in male rats. In light of our recent findings that diabetic female rats develop greater HT, the current study was designed to test the hypotheses that: 1) cerebral microvascular MMP3 activation contributes to poor functional outcomes and increased hemorrhagic transformations (HT) after ischemic stroke, and 2) MMP3 inhibition can improve functional outcomes in female diabetic rats. Female control and diabetic Wistar rats were subjected to 60 min of middle cerebral artery occlusion (MCAO). One cohort of diabetic animals received a single dose of MMP3 inhibitor (UK356618; 15mg/kg; iv) or vehicle after reperfusion. Neurobehavioral outcomes, brain infarct size, edema, HT, and MMPs were measured in brain tissue. Diabetic rats had significant neurological deficits on Day 3 after stroke. MMP3 expression and enzyme activity were significantly increased in both micro and macro vessels of diabetic animals. MMP3 inhibition improved functional outcomes and reduced brain edema and HT scores. In conclusion, cerebral endothelial MMP3 activation to vascular injury in female diabetic rats. Our findings identify MMP3 as a potential therapeutic target in diabetic stroke.

Keywords

Diabetes; Stroke; MMP3; endothelial cells; Brain

Corresponding Author: Adviye Ergul, MD, PhD, 171 Ashley Ave. MSC 908, Department of Pathology & Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425, Tel: 1-843-876-5958. ergul@musc.edu.

Author Statement

All persons who meet authorship criteria are listed as authors they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

Declaration of Competing Interest

Authors declare that competing interest is none.

1. Introduction

Stroke is a leading cause of mortality and long-term disability worldwide. Men are at a higher risk of stroke, but this fact reverses in older age. Unfortunately, death and disabilities due to stroke are greater in women (Appelros et al. 2009; Reeves et al. 2008; Roquer et al. 2003). This epidemiology is changing with the incidence of ischemic stroke increasing in younger female populations due to the increase in pre-existing risk factors like hypertension, obesity, and diabetes (Persky et al. 2010). Diabetes increases the risk of ischemic stroke by 2–6-fold (Baird et al. 2003). Experimental studies have complimented the clinical outcomes by demonstrating that diabetes exacerbates vascular injury including edema, hemorrhagic transformation (HT), and blood brain barrier (BBB) disruption causing poor outcomes and recovery, especially in females (Abdul et al. 2019; Bejot and Giroud 2010; Ergul et al. 2007; Ergul et al. 2012; Li et al. 2013; Li et al. 2019; Li et al. 2017; Vannucci et al. 2001; Zhang et al. 2004). Thus, understanding of sex-specific vascular contributions to injury is important and will help in defining therapeutic strategies.

Matrix metalloproteinases (MMPs) are zinc-binding endopeptidases that participate in both injury and repair processes after ischemic stroke (Yang et al. 2013). MMP2 and MMP9 are the most extensively studied MMPs implicated in mediating HT after stroke in both clinical and preclinical studies (Lenglet et al. 2015; Yang et al. 2011a). However, MMP3 was reported to be a critical mediator of tPA-induced HT (Suzuki et al. 2007). Moreover, our group has also previously shown perivascular expression of MMP3 was increased and pharmacological inhibition or genetic ablation of MMP3 reduced HT while improving short-term functional outcomes after ischemic stroke in acute hyperglycemia (Hafez et al. 2016). Since diabetic females are at a greater risk of HT after ischemic stroke, the current study was designed to further define the role of MMP3 in diabetic female animals. We hypothesized that activation of microvascular MMP3 mediates post-stroke HT and inhibition of MMP3 improves poor functional outcomes in female diabetic rats.

2. Materials and Methods

2.1 Animals

Animal studies were conducted at the Augusta University in Augusta, GA (Cohort 1) and Ralph H. Johnson Veterans Affairs Health Care System in Charleston, SC (Cohort 2). Animal care facilities at both institutions are approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were conducted following the National Institute of Health (NIH) guidelines for the care and use of animals in research and all the experimental protocols were approved by the institutional animal care and use committees. Studies adhered to the current ARRIVE guidelines. All behavioral testing and data analyses were conducted in a blinded manner. Control and diabetic animals were fed standard rat chow or a 45% high-fat diet (HFD), respectively. They were supplied with tap water *ad libitum* and maintained at 12 hours of light/dark cycle (6 am/6 pm). The animals were randomly assigned to groups without consideration of the estrus cycle stage.

2.2 Induction of Diabetes

Diabetes was induced in female Wistar rats (Envigo RMS, Inc., Indianapolis, IN) using an HFD/low dose streptozotocin (STZ) combination as described previously (Abdul et al. 2019; Li et al. 2019). Briefly, after 2 weeks of HFD, a single dose STZ injection (35 mg/kg; Cayman Chemical, Ann Arbor, MI) was administered intraperitoneally at 6 weeks of age. A booster dose (20 mg/kg) was given 5 days later if blood glucose was below 150 mg/dL. Control rats were maintained on regular chow with 4% kcal fat. Body weight and blood glucose were measured twice a week until euthanasia. Baseline body weight and blood glucose before middle cerebral artery occlusion (MCAO) are presented in Table 1.

2.3 Study Design

Experiments were performed in two cohorts of animals. In the first cohort, control (n=16) and diabetic (n=19) animals underwent MCAO surgery. A greater number of diabetic animals were included due to the well-established greater mortality rate in diabetic animals. On Day 1, animals who showed circling behavior and an adhesive removal test (ART) score higher than baseline were included in the study. On Day 3 post MCAO, after the evaluation of neurobehavioral outcomes, brain micro and macro vessels were isolated for vascular MMP3 analysis (n=6/group). The brain tissue from the rest of the animals (n=6/group) was used for whole-brain MMP analysis by PCR and immunofluorescence. Experiments in the second cohort of the study were planned based on the results of the first cohort and only diabetic animals were used. Post MCAO, diabetic animals were randomized to vehicle or MMP3 inhibitor (UK356618; 15mg/kg; iv; cat# 4187; Tocris Biosciences) treatment (n=11/group). The drug was intravenously injected through the jugular vein (15mg/kg) at reperfusion and the dose was based on our previous study (Hafez et al. 2016). The number of animals that entered and completed the study are shown in Fig. 1.

2.4 MCAO Surgery

Stroke was induced by transient (60 min) MCAO at 12–15 weeks of age as previously described (Abdul et al. 2019). Briefly, under 2% isoflurane anesthesia, a midline cervical incision was made to expose common, external, and internal carotid arteries. A rounded tip 3–0 monofilament nylon suture was inserted into the external carotid artery and advanced into the internal carotid artery to occlude the origin of the MCA. The occlusion suture was secured with a silk suture at the external carotid artery. After 60 min of occlusion, the suture was gently removed to allow reperfusion. In the postoperative period, blood glucose was monitored daily.

2.5 Assessment of Neurovascular Injury

Animals were put into deep anesthesia using isoflurane, and intra-cardiac perfusion was performed with cold PBS to flush out the blood cells from cerebral vessels. Brains were isolated and sliced into 6 coronal sections (A–F) of 2 mm thickness. Infarct size was measured after 2,3,5-triphenyltetrazolium chloride (TTC) staining and edema was calculated as a percent increase in the size of the ischemic hemisphere vs. the contralateral hemisphere as previously described (Ergul et al. 2007; Kelly-Cobbs et al. 2013). Macroscopic HT was

measured in sections B to E using a four-point rubric and the total score for each animal was reported.

2.6 Neurobehavioral Outcome Measurements

Neurobehavioral tests were assessed and recorded at baseline before ischemic stroke surgery and on day 3 after ischemic stroke surgery in a blinded fashion (Abdul et al. 2019; Li et al. 2017). Briefly, before behavior testing, animals were handled for 5–7 days. Bederson's score was obtained by using multiple parameters which include circling bias, hind limb retraction, forelimb flexion, and resistance to push. A maximum score of 7 is given to a normal rat in the above tests. Beam walk scores were obtained from beam walking ability and graded from 7 to 0 (7 as normal walking and 0 as an inability to balance on beam). A total composite score was presented as the sum of Bederson's score and beam walk score (maximum 14) with higher scores indicating better outcomes. ART was used to assess fine sensorimotor functions. Contact and removal latency of an adhesive paper dot was recorded on Day 3 after MCAO. For each day, the average was taken from 3 trials with a maximum removal latency of 180 seconds per trial. Higher scores indicated greater sensorimotor deficits.

2.7 Cerebral Microvessel Isolation

Microvessels were isolated with slight modifications as previously described (Hafez et al. 2016). Briefly, animals were anesthetized, decapitated and brain tissue was removed from the skull. Meninges and choroid plexuses were removed from brain tissue and cerebral hemispheres were dissected out. The hemispheres were cut into small pieces using dissection scissors in a Petri dish. Then the tissue is homogenized in a five-fold volume of phosphate-buffered saline (PBS) and centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 15 ml of PBS and mixed with an equal volume of 30% dextran, and centrifuged for 10 min at 4000 rpm at 4 °C. The top myelin containing dense white layer is removed. The pellet was re-suspended in PBS and passed through a 100-μm mesh. The filtrate was collected by washing the mesh with PBS in a 50 ml tube followed by centrifugation for 10 min at 3000 rpm at 4 °C, and the resulting pellet was re-suspended in 0.2 ml of RIPA buffer in a 1.5ml centrifuge tube. Each tube was sonicated using a 3 sec pulse three times on ice. Tubes were again centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was collected and prepared for MMP3 enzymatic activity evaluation and Western blot analysis.

2.8 In Vitro MMP3 Studies with Human Female Brain Microvascular Endothelial Cells (HBMECs)

hCMEC/D3 cell line derived from a female patient was a kind gift from Dr. J. Zastre at the UGA College of Pharmacy. Cells were cultured in 75 cm^2 culture flasks that were coated with 0.2% w/v gelatin (porcine Type A; Sigma-Aldrich) before cell seeding. A 1:1 ratio of endothelial growth media (VEC Technologies, Rensselaer, NY, USA) and Medium 199 (Corning, Manassas, VA, USA) was used for cell culture. To mimic diabetes-like conditions, cells were split into two groups: normal glucose (NG;1mM glucose) and high glucose/palmitate (HG, 25 mM glucose/P, 50 μM) in 1:1 ratio in VEC: M199 media. The cells were incubated for two days, with the media being replaced after the first day. On the

third day, all cells were starved in Dulbecco's Modification of Eagle's Medium (DMEM; Corning, Manassas, VA, USA) containing 1% penicillin-streptomycin without serum. After a six-hour of starvation, hypoxia was induced by treatment with 200 μM cobalt(II) chloride hexahydrate for 12 hours (Abdul et al. 2020; Miyamoto et al. 2015; Wu and Yotnda 2011). Media was collected for MMP3 activity measurement and cells were collected for qRT-PCR analysis of MMPs.

2.9 Evaluation of MMP3 Enzyme Activity

MMP3 enzymatic activity in isolated microvessels and HBMECs was determined using a fluorescence resonance energy transfer peptide and immunocapture assay as described previously (Hafez et al. 2016). Briefly, brain microvascular homogenates or cell lysate (100 μL) was incubated with 2 mol/L 5-FAM/QXL 520 fluorescence resonance energy transfer peptide (cat no. 60580–01; AnaSpec, San Jose, CA) in assay buffer in a 96well plate for 8 hours at 37°C, then relative fluorescence units were read at excitation/emission wavelengths of 485 of 528 nm, respectively, in a Synergy HT multimode microplate fluorescence reader (BioTek, Winooski, VT). Data was presented as percent of fluorescence intensity from the contralateral side.

2.10 Evaluation of MMP2 and MMP9 Enzyme Activity

MMP2 and MMP9 activity in HBMECs was evaluated in the concentrated medium. The medium was subjected to 10% SDS-PAGE on precast zymogram gel (BioRad) under nonreducing conditions. After electrophoresis, gels were washed twice in 50 mM Tris-HCl buffer (pH 7.5) containing 2.5% Triton X-100 for 30 minutes followed by incubation in activation buffer (50 mM Tris-HCl, pH 7.5 containing 10 mM CaCl2) for 18 hours at 37°C to allow enzymatic degradation of the substrate. Gels were stained with Coomassie blue and then destained. Digestion of the substrate (gelatin) at the position of the enzyme was observed as a clear area in the otherwise uniformly dark-staining gel. The intensity of digested areas was measured by densitometry and normalized with total cellular protein.

2.11 Quantitative Real-Time PCR (qRT-PCR)

Brain tissue and endothelial cells were lysed in RNA lysis buffer and RNA was isolated using SV Total RNA isolation system (Promega, USA). The quality and quantity of extracted RNA were assayed using a Nanodrop instrument (NanoDrop Technologies, Wilmington, DE). iScript cDNA synthesis kit (cat #1708891, BioRad, Foster City, CA) was used to reverse transcribe equal quantities of total RNA following the manufacturer's instructions. Primers were custom designed from Invitrogen (Thermo Fisher Scientific). The sequences of primers used in the study are listed in Table 2. qRT-PCR was performed using iScript Reverse Transcription super mix (cat #1708840, Biorad, Foster City, CA) and StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) as per the manufacturer's protocol. The relative gene expression was analyzed by the delta-delta Ct method using GAPDH as an endogenous control gene and normalized to the respective control group.

2.12 Western Blot Analysis

Brain tissue homogenate was assessed for endothelial membrane integrity markers. Briefly, equivalent amounts of tissue sample (15 μg protein/lane) were loaded onto 10% SDS-PAGE, proteins separated, and proteins transferred to nitrocellulose membranes. After blocking the membranes with 5% bovine serum albumin, membranes were incubated overnight at 4°C with primary antibody MMP3, anti-occludin-1, and anti claudin5 at 1:1000 dilution or anti-β-actin at 1:30000 dilution. After washing, membranes were incubated for 1 hour at room temperature with appropriate secondary antibodies (horseradish peroxidase [HRP]-conjugated; dilution 1:5000). For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent reagent and the signals were monitored on Amersham imager 680 (GE Healthcare Bio-Sciences Corp., Marlborough, MA). Relative band intensity was determined by densitometry on Image-J and normalized with β-actin protein.

2.13 Immunofluorescence

Paraformaldehyde fixed frozen brain sections were subsequently washed with TBS followed by treatment with 0.2% Triton X-100 for 3 minutes. After washing, samples were blocked by 5% BSA for 1 hour at room temperature. Sections were then incubated with primary antibodies like anti-MMP3, anti-CD31, anti-GFAP, and anti-NeuN at a 1:100 dilution in 0.2% BSA at 4°C overnight. Cells were washed and incubated with AlexaFlour 488 and Alexa 595 conjugated secondary antibodies (Jackson Immuno Research Laboratories, Inc., West Grove, PA) at a 1:500 dilution at room temperature for 1 hour. Negative control slides were incubated with 0.2% BSA in place of the primary antibody. Slides were imaged on Axiovert 200 microscope (Carl Zeiss MicroImaging, Thornwood, NY).

2.14 Data Analysis

Power analysis was made at alpha=0.05. Based on data for HT in our past studies, a sample size of 8/group was predicted to provide at least 85% power to detect the effect of disease, and 40% extra animals were added due to the increased mortality with diabetes. Unpaired t-tests were used to compare the two groups. Data were expressed as Mean ± SEM. $p<0.05$ was considered significant. One-way ANOVA and Tukey's post hoc comparisons were used to compare multiple groups of cell culture data except for Fig. 4C, 4D, and 4E which were analyzed by 2-way ANOVA (NG × HG-P) × (normoxia × hypoxia).

3. Results

3.1 Effect of ischemic stroke on functional outcomes and MMP3 expression in female diabetic rats

Diabetic animals experienced greater neurological deficits on Day 3 after ischemic stroke as indicated by lower composite scores (Fig. 2A) and higher adhesive removal time (Fig. 2B) as compared to controls. Expression of MMP3 protein in brain homogenates was significantly increased in the ipsilateral side when compared to the respective contralateral side (Fig. 2C) in both control and diabetes. MMP3 activity measured in isolated brain microvessels was also significantly greater in diabetic animals than in control rats (Fig. 2D). Ipsilateral brain tissue mRNA analysis showed increased expression of MMP2, MMP3, and

MMP9 by 80, 380, and 40%, respectively, (Fig. 3A) in diabetic animals. Brain macrovessels (MCA) showed a 2 and 20-fold increase in MMP9 and MMP3 expression, respectively, after stroke in diabetic animals as compared to controls (Fig. 3B). Immunofluorescence imaging of brain sections demonstrated greater MMP3 expression in diabetic animals that colocalized with endothelial cell (CD31) and neuronal (NeuN) markers whereas colocalization with astrocytes was sparse (Fig. 4A–C).

3.2 Effect of hypoxia and diabetes on MMPs in HBMECs

HBMECs cultured in normal or diabetes-mimicking conditions were subjected to hypoxic conditions induced by $CoCl_2$. Hypoxia increased the mRNA expression of MMP3 and MMP9 many folds in both groups but cells cultured in diabetic conditions showed a much greater increase in the expression of MMP2, MMP3, and MMP9 genes (Fig. 5A & B). MMP2, MMP3, and MMP9 enzyme activity measured in media collected after hypoxia exposure alone were not increased. Interestingly, cells cultured in diabetic conditions had a significant increase in MMP activity with or without hypoxia compared to cells in normal conditions (Fig. 5C–E).

2.13 Effect of MMP3 inhibition on post-stroke ischemic injury in diabetic females

Early inhibition of MMP3 did not reduce the infarct size of diabetic animals, however, edema and macroscopic HT were significantly reduced ($p<0.001$ and $p<0.05$ respectively; Fig. 6 A–D). MMP3 inhibition did improve the composite score, however, it did not reach significance (Fig. 7A). Fine motor functions measured by ART were significantly improved in animals treated with MMP3 inhibitor ($p<0.05$; Fig. 7B).

2.14 Effect of MMP3 inhibition on MMPs and tight junction proteins

Brain homogenate from the ipsilateral side of the brain was used to analyze the mRNA expression of MMPs and tight junction proteins. There were no changes in the mRNA expression of MMP2, MMP3, or MMP9 with the treatment of MMP3 inhibitor UK356618 (Fig. 8A). However, mRNA expression of ZO-1 and claudin5 was higher in animals treated with MMP3 inhibitor (Fig. 8B). Further, protein levels of occludin1 and claudin5 were also significantly higher in animals treated with MMP3 inhibitor (*, $p<0.05$; Fig. 8C).

4. Discussion

It is well established that diabetes increases the risk of ischemic stroke and worsens functional outcomes There is also emerging evidence that diabetes increases the incidence of stroke in younger individuals, especially females (Baird et al. 2003; Goldenberg et al. 2022; Persky et al. 2010; Rexrode et al. 2022; Sifat et al. 2022; Simats and Liesz 2022). Clinical and preclinical studies, which used predominantly male animals, corroborated exacerbated cerebrovascular injury characterized by greater HT and edema after stroke in diabetes (Abdul et al. 2019; Ergul et al. 2007; Ergul et al. 2012; Hesami et al. 2015; Inagawa 2007; Jiang et al. 2021; Li et al. 2013; Shukla et al. 2017). Neuroprotection typically seen in young female animals is lost if animals are diabetic and they also develop greater HT compared to male animals (Li et al. 2019; Li et al. 2017). Thus, the current study focused on defining the underlying mechanisms associated with vascular injury in females.

MMPs are a big family of zinc-peptidases that can degrade many extracellular matrix proteins and contribute to both repair and injury processes (Yang et al. 2013). Various MMPs are known to be involved in the complex pathophysiology of the ischemic stroke (Candelario-Jalil et al. 2009; Jin et al. 2010; Yang et al. 2007; Yang et al. 2013). Both clinically and preclinically, MMP2 and MMP9 are the most extensively studied MMPs in post-ischemic stroke injury and have been implicated in HT (Castellanos et al. 2003; Clark et al. 1997; Lakhan et al. 2013; Rosell et al. 2006; Sole et al. 2004). Pharmacological inhibition of MMP2 and MMP9 reduced HT and improved the recovery (Fagan et al. 2010; Jin et al. 2010; Lakhan et al. 2013; Switzer et al. 2011). MMP9 inhibition by minocycline showed promising outcomes in Minocycline to Improve Neurological Outcomes in Stroke (MINOS) trial (Fagan et al. 2010; Switzer et al. 2011). These enzymes are activated in diabetes. We reported that diabetes-mediated upregulation of MMP9 promotes cerebrovascular remodeling in diabetic Goto-Kakizaki rats, which develop HT if an ischemic injury is overlayed on this pathology (Elgebaly et al. 2010). Moreover, glycemic control or inhibition of MMPs by minocycline prevented cerebrovascular remodeling and associated HT. In the current study, we observed both MMP2 and MMP9 activity were increased in cell culture experiments but in the vasculature or brain tissue homogenates, the increase in MMP3 expression was the most robust.

While it is not as widely studied as MMP2 and MMP9, there is evidence that MMP3 expression is increased after ischemic stroke in diabetes (Candelario-Jalil et al. 2009; Hawkins et al. 2007). The inducible form of MMP3 is found to be upregulated in the postmortem brain specimens of patients who died of a stroke. MMP3 can target and degrade many extracellular matrix and tight junction proteins (Sole et al. 2004; Yang et al. 2013). Using knock-out animals, Suzuki and colleagues showed that MMP3, and not MMP9, is the major mediator of tissue plasminogen activator (tPA)-induced HT (Suzuki et al. 2007). Our group has shown that acute hyperglycemia increases neurovascular injury and the use of tPA in hyperglycemic conditions worsens stroke outcomes (Hafez et al. 2014). We have also shown that genetic modulation or pharmacological inhibition of MMP3 improves functional outcomes (Hafez et al. 2016). Based on these premises, the current study was designed to elucidate the role of MMP3 in ischemic injury in female diabetic animals. We observed a significant increase in the expression of MMP3 protein as well as MMP3 enzyme activity in brain microvasculature. MMP3 gene expression measured in total brain tissue and macro vessels (MCA) was also many folds higher in the ischemic side of the brain and it was similar to the previous observation in the hyperglycemia (Hafez et al. 2016). Immunofluorescence showed colocalization of MMP3 with neurons and endothelial cells (Yang et al. 2011b). Many cell types within the neurovascular unit express MMP3, but considering the BBB disruption in ischemic injury, microvasculature appears to be a key target (Yang et al. 2013). Previous studies have also shown the impact of MMP3 on brain endothelial cells but the sex of the cells was not always reported (Hummel et al. 2001; Lee et al. 2014; Liu et al. 2013). We used human female BMVECs, which exhibited greater MMP2, MMP3, and MMP9 gene expression when cultured in normal or diabetic conditions and subjected to hypoxia. The increase in MMP gene expression was multi-fold higher in cells cultured in diabetic conditions. Cells cultured in diabetic conditions had greater MMP3 activity compared to control, but hypoxia did not further amplify it in any of the conditions.

While we observed MMP3 mostly in the vasculature and to some extent in neurons, the role(s) of neuronal or glial MMP3 in neurovascular injury remains to be determined to fully understand the underlying mechanisms.

MMP enzymes play in a consortium to regulate the injury and repair process (Lakhan et al. 2013). tPA the only thrombolytic drug available for use has a limited time window and has side effects like edema and hemorrhages due to activation of MMPs. Thus, a combination of tPA and MMP inhibition could limit the side effect and extend the treatment time window in ischemic stroke, especially in patients with comorbidities like diabetes (Fan et al. 2014). Long-term inhibition may be detrimental as MMPs are needed in the repair process (Wang et al. 2008). However, there is limited information focusing on MMP3 inhibition post-ischemic stroke with comorbid conditions like diabetes, especially in females. Outcomes, of previous studies by Hafez et al, in male hyperglycemic rats, encouraged us to extend the current study to diabetic females and use the pharmacological inhibitor UK356618 for the MMP3 inhibition (Hafez et al. 2016). One caveat of the current study is that we did not monitor the estrus cycle of the animals and both control and diabetic rats were randomly assigned to MCAO surgery. Our decision was based on our recent study that there was no difference in the severity of stroke injury and functional outcomes when 60-min MCAO was performed in rats in the proestrous stage or in ovariectomized female rats (Eldahshan et al. 2019). Additionally, earlier studies showed that greater sensitivity to ischemic injury may be more pronounced in animal models with comorbid disease conditions (Carswell et al. 2000a; Carswell et al. 2000b). Nevertheless, numerous preclinical studies showed that ischemic injury is exacerbated by estrogen depletion and our findings need to be validated in animals in which the estrus cycle is carefully monitored as well as in older animals.

In conclusion, our key findings are that 1) female diabetic animals suffer from poorer functional outcomes after stroke than control female animals, 2) enhanced expression of MMP3 is observed in the cells of the neurovascular unit, 3) the MMP3 activity measured in the brain microvasculature is significantly increased the diabetic animals, 4) the pharmacological inhibition of MMP3 improves functional outcomes and reduces vascular injury, and 5) MMP3 inhibition improves the expression of tight junction proteins occluding-1 and claudin-5. Thus, the improvement in edema and HT could be attributed to improved membrane integrity of microvasculature due to the inhibition of MMP3. The use of only female animals could be considered a limitation of the study; gathering gender-specific information will help determine tailor-made targeted therapeutic strategies to improve post-stroke recovery. In conclusion, post-stroke early inhibition of MMP3 has therapeutic potential in females with diabetes.

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HIGHLIGHTS

- Female diabetic animals suffer from poorer functional outcomes after stroke than control female animals.
- Enhanced expression of MMP3 is observed in the cells of the neurovascular unit.
- The MMP3 activity measured in the brain microvasculature is increased the diabetic animals.
- The pharmacological inhibition of MMP3 improves functional outcomes and reduces edema and hemorrhagic transformation (HT).
- MMP3 inhibition improves the expression of tight junction proteins.

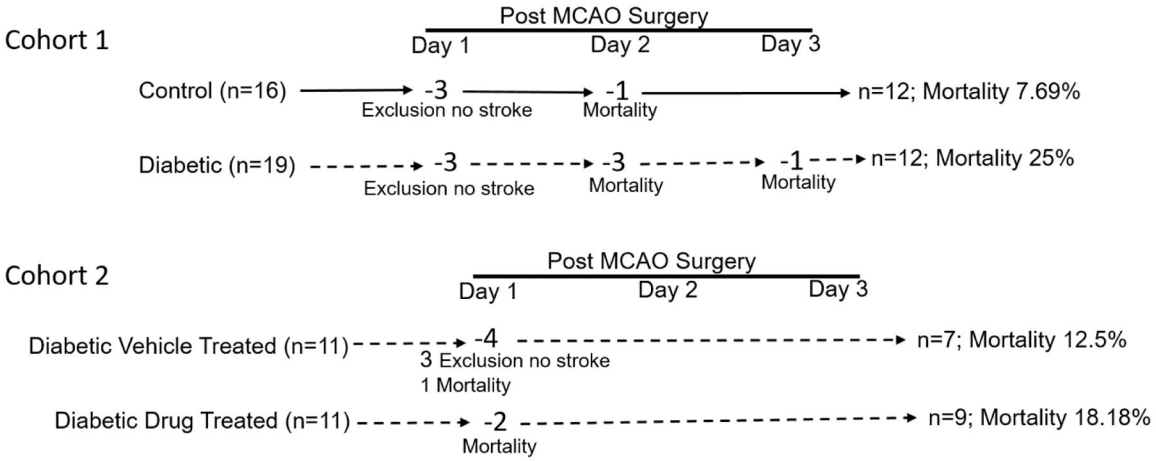


Fig. 1.

Schematic timeline and description of the experimental design, animal numbers per group, and mortality rate.

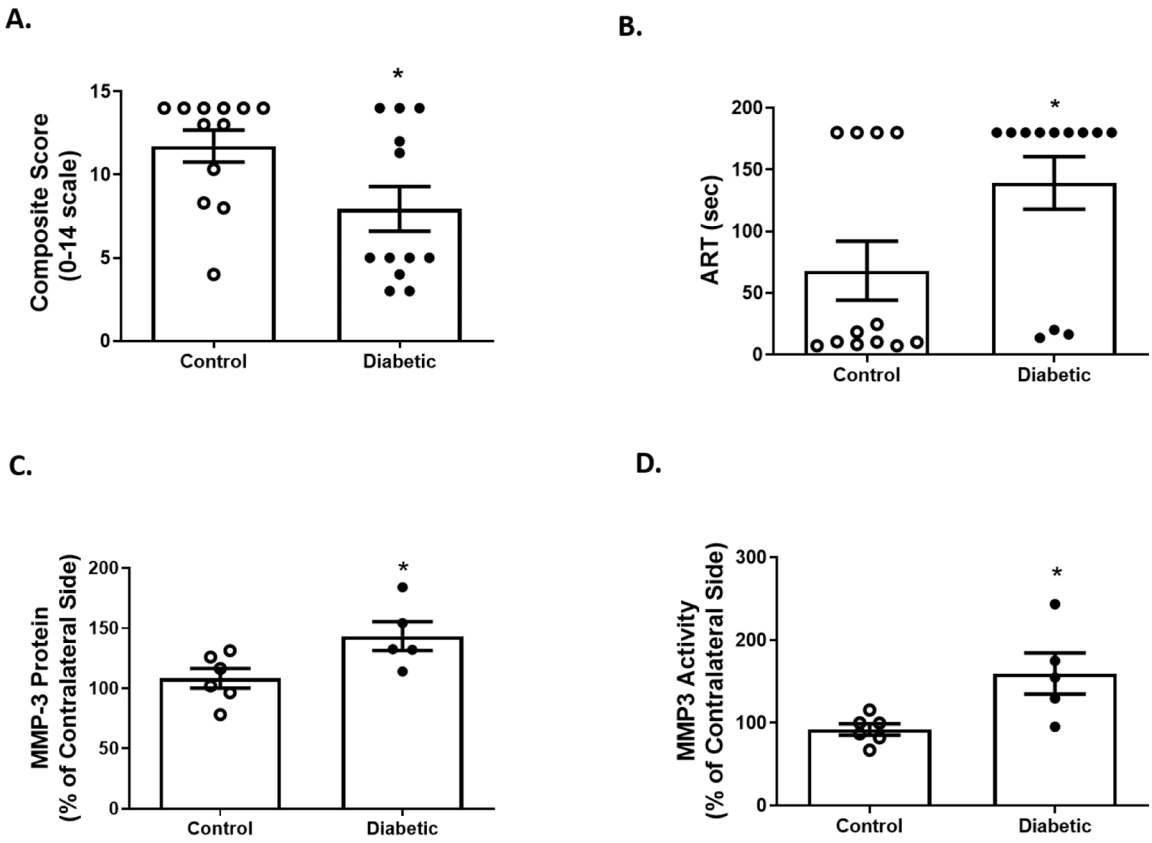


Fig. 2.

Ischemic stroke in female diabetic animals worsens behavioral outcomes and increases MMP3 in brain microvessels. Measurement of the composite score (A) and adhesive removal test (B) was performed 3 days after ischemic stroke in control and diabetic female rats. (*, $p<0.05$, vs controls group; n=12). Expression of MMP3 protein (C) and MMP3 enzyme activity (D) in isolated brain microvessels was significantly higher in diabetic animals compared to control (*, $p<0.05$; n=5–6 in each group).

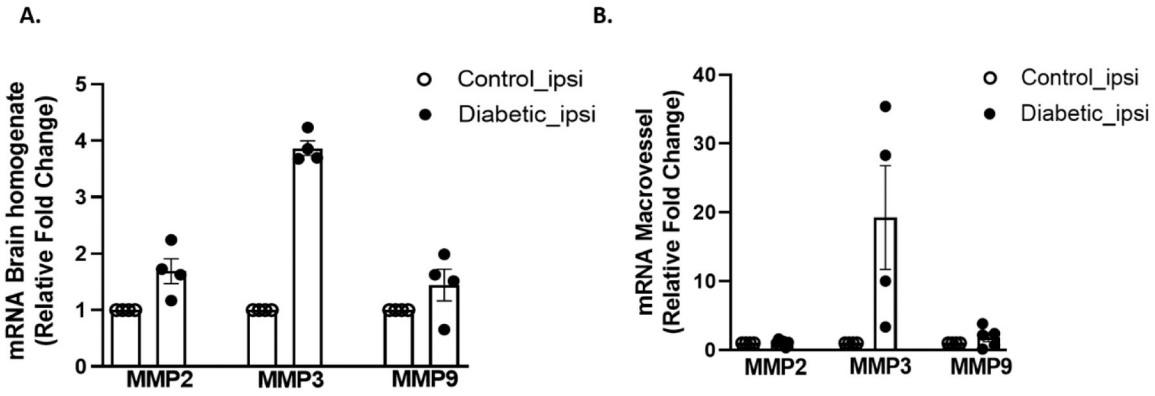
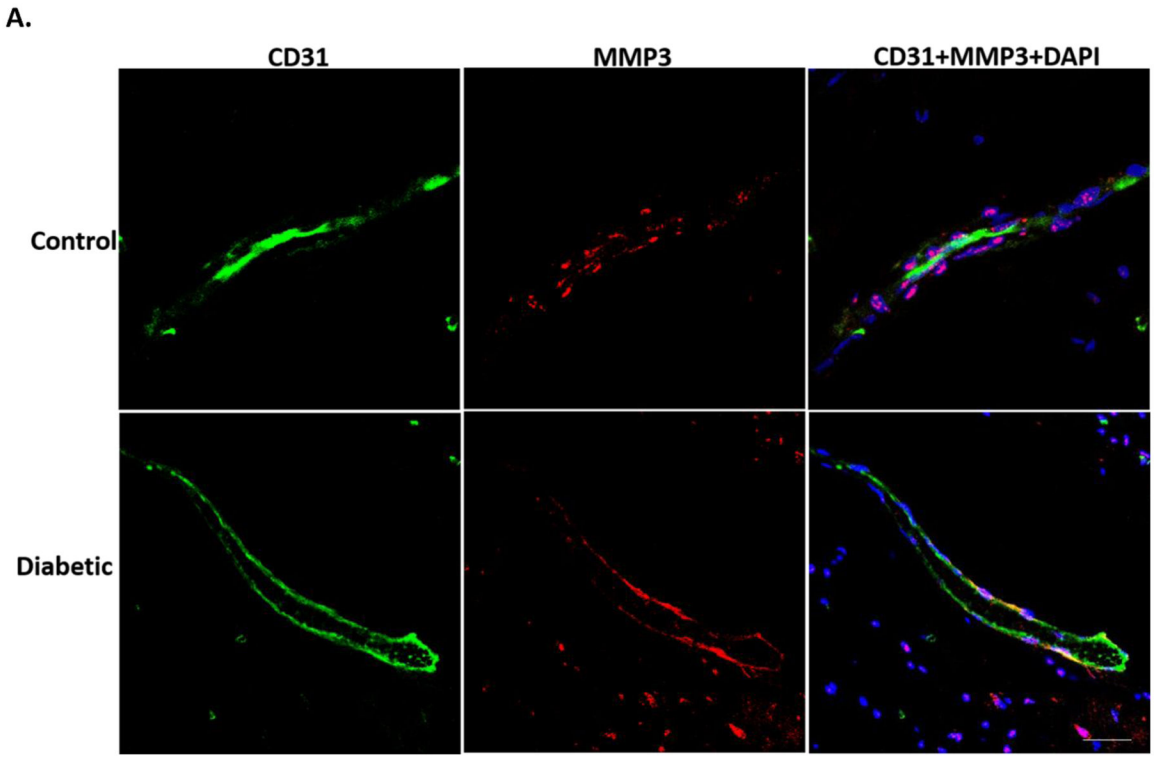


Fig. 3.

Ischemic stroke increased the expression of MMP genes in the brain. (A) mRNA expression of MMP2, MMP3, and MMP9 measured in the ipsilateral side of the brain was robustly increased in diabetic animals compared to control animals. (B) MMP3 and MMP9 were also expressed in brain macrovessels (MCA) of diabetic animals compared to control animals (n= 4–6 in each group).



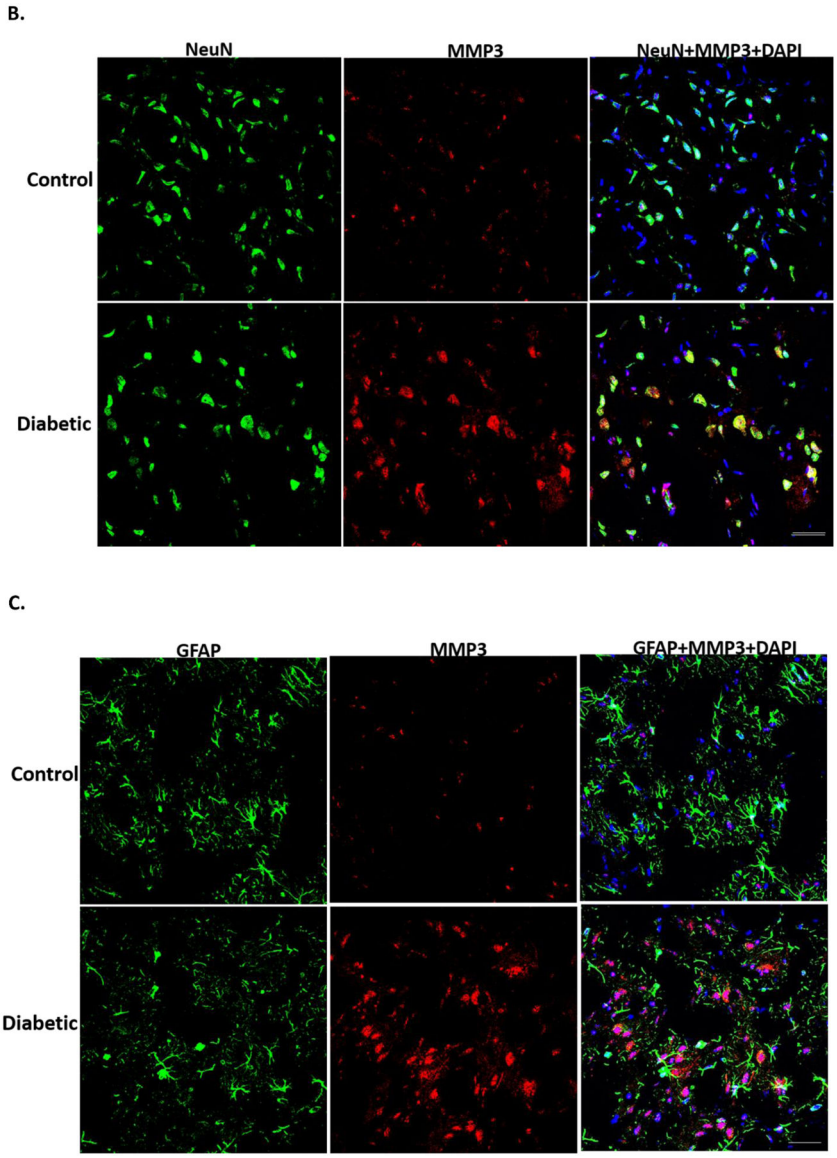


Fig. 4.

Representative images of immunofluorescence showing MMP3 expression in the cortex of the ischemic hemisphere 3 days after ischemic stroke. MMP3 immunoreactivity was greater in diabetic animals. MMP3 is colocalized with endothelial cell marker CD31 (A) and neuronal marker NeuN (B). While MMP3 expression was more around astrocyte cell marker GFAP (C). Images were captured at 20x magnification and the scale bar is 25 μm (n=4 in each group).

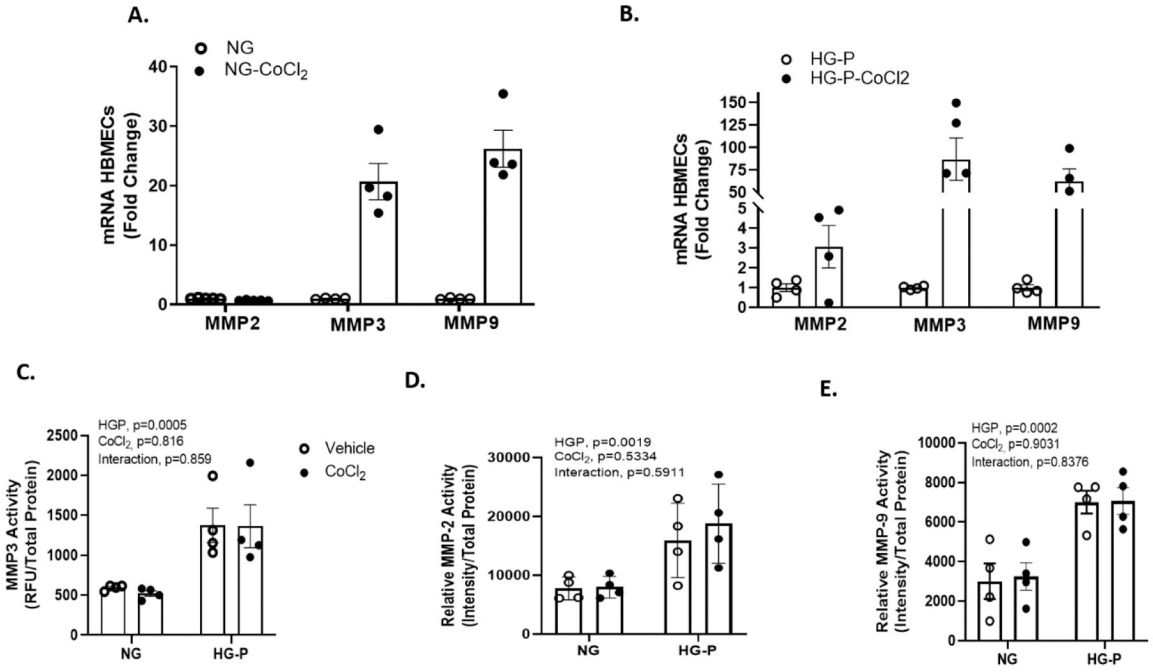


Fig. 5.

Hypoxia activates MMPs in female HBMECs in diabetic conditions. Hypoxia ($CoCl_2$) increased the mRNA expression of MMP2, MMP3, and MMP9 (A) in both normal (NG) and diabetic (HG+ P; B) conditions. interestingly, this increase was much higher in diabetic conditions compared to normal conditions (n=4–5). MMP3 enzyme activity (C) measured by FRET assay and MMP2 and MMP9 (D & E) activity measured by zymogram was significantly increased in media collected from cells in diabetic conditions, however, hypoxia did not increase it further (n=4).

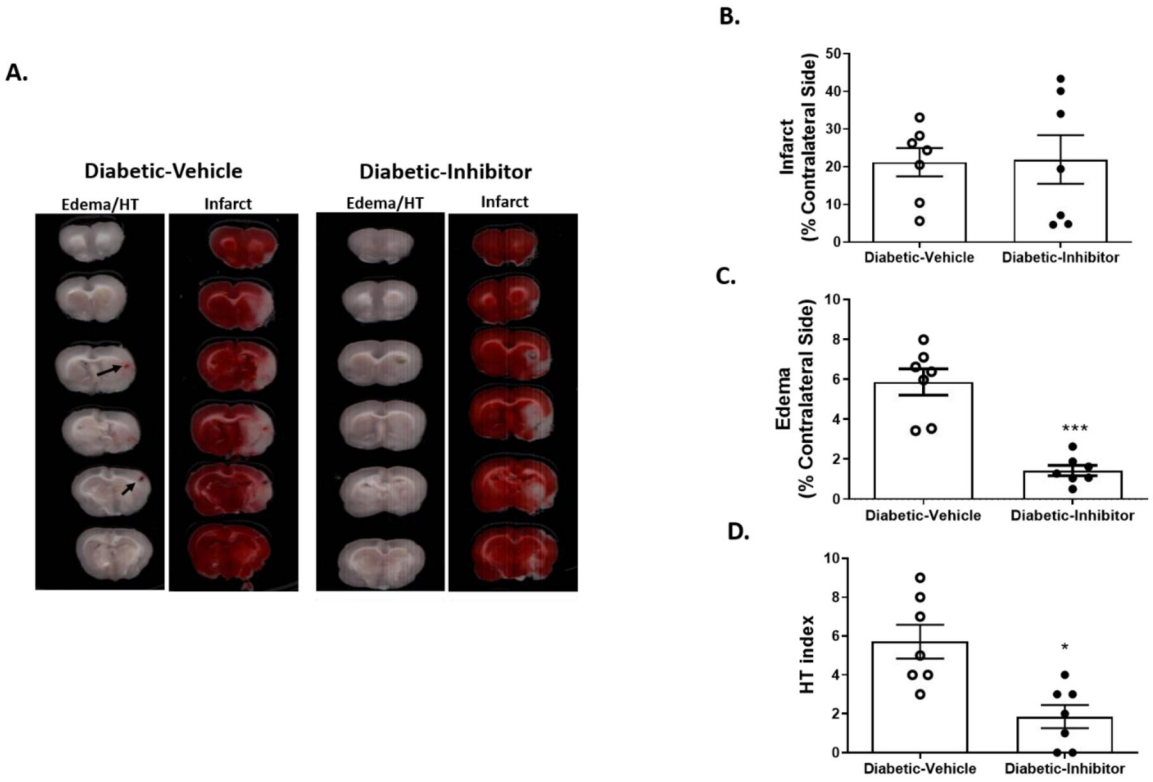


Fig. 6.

Inhibition of MMP3 reduces the neurovascular injury in female diabetic animals subjected to ischemic stroke. Female diabetic rats were subjected to 60 min of MCAO and treated with vehicle or MMP3 inhibitor UK356618 (15 mg/kg; i.v.) at reperfusion. After 72 h of reperfusion, animals were sacrificed to measure the neurovascular injury. (A) Representative images showing edema and infarct (arrows indicate the hemorrhages). (B) Treatment with an MMP3 inhibitor did not show any difference in infarct size. (C) Edema was significantly reduced in animals treated with MMP3 inhibitor (***, $p<0.001$). (D) Hemorrhagic transformation (HT) index was significantly reduced in animals treated with MMP3 inhibitor compared to vehicle-treated animals (*, $p<0.05$; n=7 in each group).

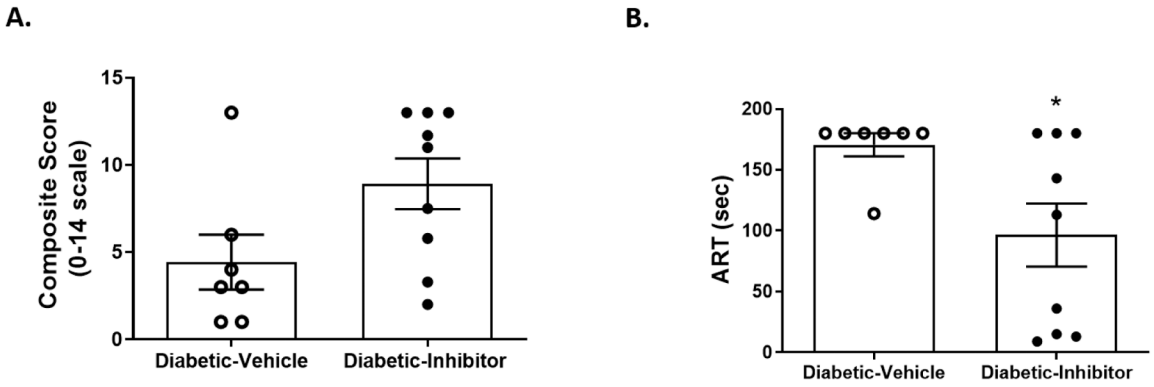


Fig. 7.

Inhibition of MMP3 improves functional outcomes in female diabetic animals after ischemic stroke. (A) The composite score measured on Day 3 post-stroke was improved in animals treated with an MMP3 inhibitor. (B) Adhesive removal time (ART) was significantly reduced in animals treated with MMP3 inhibitor (*$p < 0.05$; n=7–9 in each group).

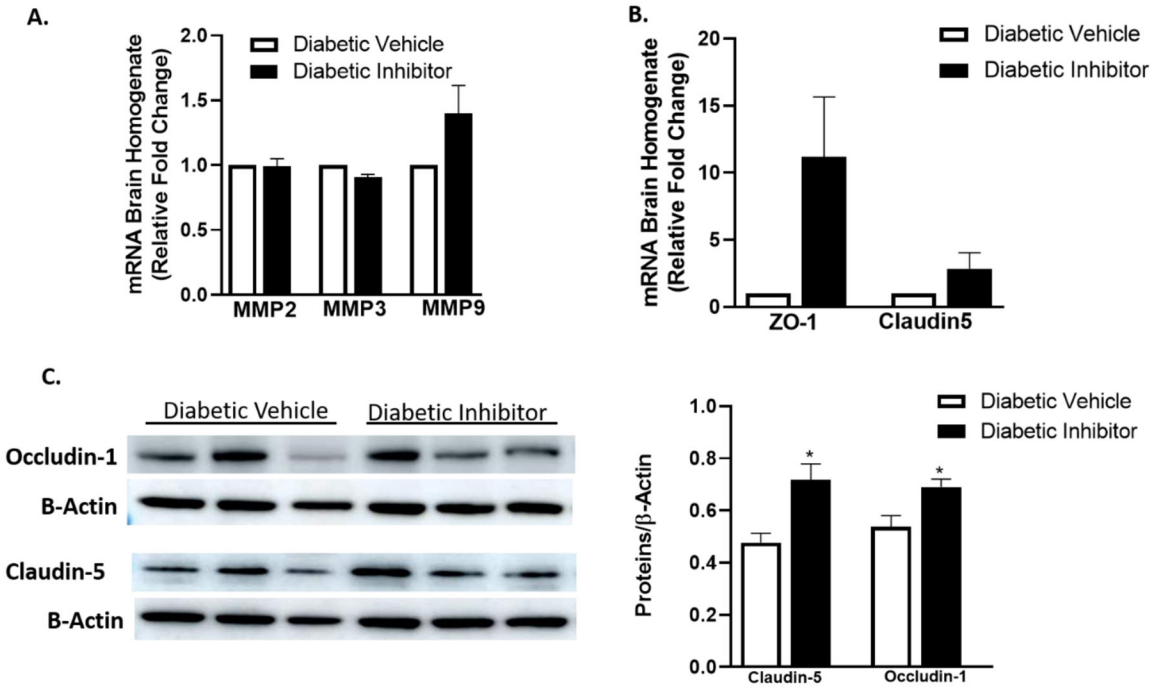


Fig. 8.

Inhibition of MMP3 after ischemic stroke improves the expression of tight junction proteins in female diabetic animals. (A) mRNA expression of MMP2, MMP3, and MMP9 was not different in the ischemic side of the brain between the vehicle and MMP3 inhibitor-treated animals. (B) mRNA expression of ZO-1 and claudin5 in the ischemic side of the brain was higher in animals treated with an MMP3 inhibitor. (C) Tight junction proteins occludin1 and claudin5 were also significantly higher in the ischemic side of the brain in animals treated with MMP3 inhibitor (*, $p<0.05$, n=6–9 in each group).

Table 1.

Body weight and blood glucose measurements

Cohort 1		
Female Wistar Rats	**Body weight (gm)**	**Blood Glucose (mg/dl)**
Control (n=16)	283.7±4.0	90.09±3.46
Diabetic (n=19)	277±15.13	346.6±31.14 ***
Cohort 2		
Female Diabetic Wistar Rats	**Body weight (gm)**	**Blood Glucose (mg/dl)**
Vehicle (n=11)	285.0±22.0	315.7±36
UK356618 (n=11)	260.8±16.12	350.0±29.43

*** p<0.0001 compared with control rats

Table 2.

PCR primer sequences

Primer Name	Forward	Reverse	NCBI Reference
Rat- MMP3	TGGGTCTCTTTCACTCAGCC	AGGGGATTCTGTGGGAGGTC	NM_133523.3
Rat- MMP9	ATGGGAGAGAAGCAGTCCCT	TGATGGTGCCACTTGAGGTC	NM_031055.2
Rat- MMP2	TGACGATGAGCTGTGGACTC	GCTGCTGTATTCCCGACCAT	NM_031054.2
Rat-GAPDH	GAAGCTGGTCATCAACGGGA	CGACATACTCAGCACCAGCA	NM_017008.4
Human-MMP3	CACAGACCTGACTCGGTTCC	AGGTTCTGGAGGGACAGGTT	NM_002422.4
Human-MMP9	CGCAGACATCGTCATCCAGT	GGACCACAACTCGTCATCGT	NM_004994.2
Human-MMP2	GGACTTAGACCGCTTGGCTT	GGGCAGCCATAGAAGGTGTT	NM_001127891.2
Human-GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC	NM_001256799.2
Human Zo-1	CGCTCAAGAGGAAGCTGTGG	GAGGGTTTTCCTTGGCTGAC	NM_001301025.3
Human- CLDN5	GGGTTTGTGTCCCTGCCTAA	CCAGTGCAAGATCCCAGAGG	NM_001363066.2