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Research Paper

Post-stroke neovascularization and functional outcomes differ in diabetes depending on severity of injury and sex: Potential link to hemorrhagic transformation

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ABSTRACT

Diabetes is associated with increased risk and worsened outcome of stroke. Previous studies showed that male diabetic animals had greater hemorrhagic transformation (HT), profound loss of cerebral vasculature, and poor behavioral outcomes after ischemic stroke induced by suture or embolic middle cerebral artery occlusion (MCAO). Females are protected from stroke until reaching the menopause age, but young females with diabetes have a higher risk of stroke and women account for the majority of stroke mortality. The current study postulated that diabetes is associated with greater vascular injury and exacerbated sensorimotor and cognitive outcome after stroke even in young female animals. Male and female control and diabetic animals were subjected to transient MCAO and followed for 3 or 14 days to assess the neurovascular injury and repair. The vascularization indices after stroke were lower in male diabetic animals with 90-min but not 60-min ischemia/reperfusion injury, while there was no change in female groups. Cognitive deficits were exacerbated in both male and female groups regardless of the injury period, while the sensorimotor dysfunction was worsened in male diabetic animals with longer ischemia time. These results suggest that diabetes negates the protection afforded by sex in young female animals, and post-stroke vascularization pattern is influenced by the degree of injury and correlates with functional outcome in both sexes. Vasculoprotection after acute ischemic stroke may provide a novel therapeutic strategy in diabetes.

1. Introduction

According to the International Diabetes Federation there are 425 million people afflicted with diabetes worldwide in 2017 and the disease burden is increasing rapidly as it is linked with multiple health complications including retinopathy, nephropathy, neuropathy, cognitive impairment and ischemic stroke (IDF, 2017; Mizrahi et al., 2010). Diabetes increases the risk of ischemic stroke by 2–6 fold and ultimately leads to worsened outcomes contributing to the leading cause of disability status of ischemic stroke (Bushnell et al., 2014; Fox, 2010). Equally important, diabetes significantly increases the mortality of stroke and chances of recurrent stroke (Benjamin et al., 2017; Kruyt et al., 2010; Wei et al., 2010). Experimental studies in various models of diabetes have found similar results showing significantly increased mortality and vascular injury including edema and hemorrhagic transformation (HT) as well as poorer functional outcomes even without infarct volume change compared to the controls (Chen et al., 2011; Cui et al., 2011; Li et al., 2013; Prakash et al., 2012; Reeson et al., 2015; Sweetnam et al., 2012; Tureyen et al., 2011; Zhang et al., 2016). Our understanding of the impact of diabetes on the stroke recovery process has emerged only in recent years, but the mechanisms underlying poor recovery in diabetes remain largely unknown. Sweetnam

Abbreviations: GK, Goto-Kakizaki; HFD, high fat diet; STZ, streptozotocin; MCAO, middle cerebral artery occlusion; HT, hemorrhagic transformation; Hb, hemoglobin; ART, adhesive removal test; NOR, novel object recognition; TTC, 2,3,5-triphenyltetrazolium chloride; FFA, free fatty acids; FITC, fluorescein isothiocyanate

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et al. showed that type 1 diabetes severely affects synaptic plasticity (Sweetnam et al., 2012). Our previous studies using Goto-Kakizaki (GK) genetically diabetic rats, a lean model of type 2 diabetes, showed poor neurological outcomes following ischemic stroke. In this model, there was dramatic loss of cerebrovasculature after stroke (Ergul et al., 2007; 2012; Kelly-Cobbs et al., 2012). It is increasingly recognized that vascular restoration should accompany neuronal repair for recovery of ischemic stroke (Ergul et al., 2014; Gorelick et al., 2017; Iadecola, 2017). The consequences of stroke incurred by patients with diabetes are likely to be associated with the metabolic changes that occur in the disease. Thus, the first goal of this study was to investigate the neurovascular repair and functional outcomes in a model of diabetes that exhibits these metabolic derangements.

Women account for the majority of stroke deaths, yet men have a higher incidence of stroke up until age 75 (Benjamin et al., 2017). Women aged 49–54 years are twice as likely as men to have a stroke (Towfighi et al., 2007). Additionally, these women have poorer functional outcomes (Di Carlo et al., 2003), worse locomotor function at 1- and 5-year follow-up (Fukuda et al., 2009), greater mental impairment, and lower quality of life compared to men (Franzen-Dahlin and Laska, 2012; Glader et al., 2003; Gokkaya et al., 2005). Sex hormones have been linked to more favorable outcomes following stroke in females and strengthening this argument is the fact that women lose this protection from stroke following menopause (Normann et al., 2009; Towfighi et al., 2007). On the other hand, it has been found that diabetes increases stroke risk, especially in younger individuals and females (Bejot and Giroud, 2010). The underlying mechanisms contributing to the increased occurrence of stroke and poor functional recovery in young females with diabetes are poorly understood. Numerous experimental studies have addressed, and continue to investigate sexual dimorphism in stroke injury and recovery. Studies found that diabetes directly increases vascular damage in the acute phase of stroke in male animals, yet there are almost no studies in the young or aged female diabetic rats (Ergul et al., 2007; Ning et al., 2012; Tureyen et al., 2011). Thus, the second goal of this translational study was to compare the impact of diabetes on cerebrovascular architecture after ischemic injury in both female and male diabetic rats.

2. Materials and methods

2.1. Study design and animal groups

Animals were housed in the Augusta University Division of Laboratory Animal Services facility. This facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Protocols regarding animal care and treatment were approved by the Institutional Animal Care and Use Committee. In addition to university procedures, this study also followed the National Institute of Health guidelines for care and use of animals in research. Any behavioral testing and data analyses occurred in a blinded manner.

Wistar rats (Envigo, Indianapolis, IN) were maintained at a constant 12 h light/dark cycle and access to food and water ad libitum. The type 2 diabetic model used was induced by a 45% high fat diet (HFD, Research Diet Inc., New Brunswick, NJ) beginning at 4 weeks of age, followed by single low-dose injection of streptozotocin (STZ; 30 mg/kg) at 6 weeks of age. Control animals were fed regular chow. Body weight and blood glucose measurements occurred twice weekly until surgery at 12–13 weeks of age. Diabetes was characterized by increased blood glucose levels, insulin resistance and/or insulin deficiency, dyslipidemia including increased adiposity, plasma free fatty acids, triglyceride and cholesterol levels. In the postoperative period (first 5 days), blood glucose was monitored daily. Confirmation of elevated blood levels was based on blood glucose measurements and hemoglobin (Hb) A1c measurement with the Bayer A1cNow system (Fisher Scientific). Three sets of experiments were conducted:

2.1.1. Experiment 1

Control and diabetic male rats were subjected to sham or 90-min mechanical middle cerebral artery occlusion (MCAO) surgery and followed for 14 days (n = 5 for sham and n = 9 for stroke groups). Three animals in the diabetic stroke and one animal in the control stroke group died before Day 3 and were not included in the results (n = 6 diabetic stroke group, and n = 8 control stroke group). A battery of sensorimotor and cognitive/memory tests were performed at Days 1, 3, 7 and 14 as described below. Additional metabolic parameters were measured using the following kits: Free Fatty Acids (FFA) HR Series NEFA-HR kits from Wako Pure Chemical (Osaka, Japan), Infinity Triglycerides and Cholesterol kits from Thermo Scientific (Middletown, VA), and Rat Insulin ELISA from Millipore (Billerica, MA).

2.1.2. Experiment 2

Control and diabetic male and female rats were subjected to 60-min MCAO and followed for 14 days (n = 8) for outcomes. In this experiment, one animal died in the male control stroke group and six animals (3 female and 3 male) died in the diabetic stroke group within 36 h after ischemia. The animals that died before Day 14 were not included in analyses. As a result, final animal numbers are: n = 8 in female control, n = 7 in male control, n = 5 in female diabetic, and n = 5 in male diabetic groups.

In these sets of experiments, after 14-day behavioral tests, animals were injected with fluorescein isothiocyanate- dextran (FITC; MW: 2,000,000; Sigma-Aldrich, St. Louis, MO) to visualize the vasculature as described below.

2.1.3. Experiment 3

Control and diabetic male and female rats were subjected to 60-min MCAO (n = 8) and followed for 3 days for sensorimotor deficits. Four animals (2 female and 2 male) died in the diabetic group within 24 h of surgery so they were not included in this study (final numbers are n = 8 female control, n = 8 male control, n = 6 female diabetic, n = 6 male diabetic). Female rats underwent surgery during the diestrus phase after careful monitoring of the estrus cycle by vaginal swab.

2.2. Stroke surgery

Focal cerebral ischemia was induced by MCAO surgery as described previously (Li et al., 2017). In all surgeries, animals were anesthetized with isoflurane using 5% at induction and 2% at maintenance in the mixture of N_2 and O_2. A nylon suture was inserted through the external carotid artery to occlude the MCA for 60 or 90 min followed by reperfusion. Laser Doppler imaging system (Perimed, North Royalton, OH) was used to confirm occlusion and reperfusion before and after the suture was removed.

2.3. Behavioral tests

Sensorimotor tests including Bederson score, beam walk, grip strength, adhesive removal test (ART), and cognitive/memory test with Novel Object Recognition (NOR) were performed on Days 1, 3, 7, and 14 in a blinded manner. Animals were trained 5–7 days prior to surgery and the baseline was taken on the final day.

Bederson score involved four parameters including: ipsilateral circling (score 2 – no circling, 1 – partial circling, 0 – continuous circling), contralateral hindlimb and forelimb retraction which measuring the ability of the animal to replace its displaced limb in an appropriate manner (score 2 – immediate replacement, 1 – replacement after minutes, 0 – no replacement), and resistance to push (score 1 or 0, depending on whether the animal exhibited any resistant to push at all). A maximum score of 7 was deemed a normal, healthy animal. Beam-walk abilities were graded based on a 7-point scale method described by Feeney (Feeney et al., 1982). The entire composite score involved the sum of the Bederson score and beam-walking score, which led to a 0–14

point scale.

Grip strength was measured with a standard grip strength meter (Columbus Instrument, Columbus, OH). The rat is gently held with their forepaws grasping the mesh under tension, attached the grip strength meter and then pulled back consistently with its tail. The digital recording obtained from 3 trials is averaged and recorded as one observation (Prakash et al., 2013b).

ART was performed as previously described (Li et al., 2017). Removal latency of the adhesive paper dot was recorded on each of the testing days following stroke with three trials that were averaged. The maximum removal latency was 180 s per trial.

In order to assess cognitive deficits, we used the NOR test (Hardigan et al., 2017; Prakash et al., 2013b). Animals were habituated to test apparatus in a grey plastic box (63 L × 38 W × 42H cm) that was layered with animal bedding for 4 days prior to baseline testing for 10 min. Objects with greater intricacy and details and similar in appearances with equal and unbiased preferences for one over the other were chosen to perform the test. On the day of testing, the rats were allowed to explore two identical objects during the A/A session for a period of 5 min. The rats were returned to their home cages for a delay/retention interval of 15 min following which the rats were confronted to A/B sessions in the consisting of 5 min, during which a novel object was paired with a familiar object used in the A/A session. These activities were recorded and a recognition index was determined by the amount of time the animal spent with the new object compared to the familiar object. All objects were cleaned after each session with 30% ethanol and the bedding was ruffled and cleaned to discard cues.

2.3.1. Assessment of cerebral vascularization (Experiments 1 and 2)

To visualize cerebral vessels, rats were injected with 500 μL of 50 mg/mL FITC-dextran via the jugular vein under anesthesia using isoflurane. FITC perfused throughout the animal for 10 min. Prior to sacrifice, plasma was obtained for metabolic analysis. Brains were processed in 4% paraformaldehyde for 24–48 h followed by 30% sucrose in PBS. Brains were sectioned using a cryostat and confocal images were obtained by the Zeiss 760 confocal microscope using 100 μm sections. Specifically, *Z*-stacked images were obtained from regions of interest in the cortex and striatum (bregma −1 to +1), where we have previously found hemorrhage in diabetic animals. In order to reduce variability and confirm the findings, an overall representation was obtained using three individual images from the ipsilateral/contralateral cortical and striatal regions (Fig. 1 A). Only ipsilateral images were taken in the sham groups. The mean value from these three separate images was calculated and each animal had a total of nine unique images analyzed from the cerebrovasculature. The *Z*-stack referenced an image size of 1.984 μm, 512 × 512 pixels, and 20× magnification. Raw images were then imported into Volocity 6.0 (Improvision, Lexington, MA) where they were 3D-reconstructed to determine the vascular volume and surface area. Vascular volume references the vascular volume ratio in comparison to total volume. Surface area represented the absolute surface area of the total vasculature. Branch density and tortuosity were determined using the open-source imaging software package FIJI. Confocal images were converted into 8-bit images and reduced to 1-pixel size to obtain a binary image. These binary images were then skeletonized to determine tortuosity. Images were not pruned in order to maintain the full degree of vessel tortuosity. The final determination of tortuosity was calculated as the ratio of longest (tracing of the distance between 2 points) and shortest (the Euclidean-straight line). FIJI was also used to determine branch density, which is the number of branch points found over the entire unit length of vessel.

2.3.2. Evaluation of neurovascular injury (Experiment 3)

Brain slices (A to G, +6.7 mm to −8 mm of bregma) were imaged for analysis of macroscopic bleeding (HT Index) and then stained with 2,3,5-triphenyltetrazolium chloride (TTC) in order to determine infarct size and edema measurements. Infarct size was determined by calculating the ischemic size percentage versus the non-ischemic side. After this imaging took place, the brain sections were homogenized in order to determine hemoglobin quantification with QuantiChrom kit (BioAssay Systems, Hayward, CA). Excess hemoglobin (Hb, μg/mg protein) was determined by subtracting sham Hb values from the ischemic hemisphere readings. HT index was scored in brain slices B through E and used a five point rubric system: 1 – Dispersed individual petechiae; 2 – Confluent petechiae; 3 – Small diffuse hemorrhage or hematoma; 4 – Large diffuse hemorrhage or hematoma. The total score for each animal was reported and this led their overall HT index score. All endpoints for neurovascular injury were measured in a blinded manner.

2.3.3. Data analysis

The results were analyzed by GraphPad Prism 7 and expressed as mean ± SEM. Vascularization data in Experiment 1 was analyzed by a two-way ANOVA for (Control vs. Diabetes) and (Sham vs. Stroke). Data in Experiments 2 and 3 were analyzed by a two-way ANOVA for (Control vs. Diabetes) and (Females vs. Males) comparisons. Behavioral recovery over 14 days was analyzed by repeated measures ANOVA. A Bonferroni adjustment was used for multiple posthoc comparisons. Significance detected by ANOVA and posthoc analyses were indicated by symbols and letters, respectively.

3. Results

3.1. Metabolic profile

The metabolic profiles of animals in Experiments 1 to 3 are given in Table 1. In all studies, diabetic animals had higher blood glucose levels in comparison to their respective control groups (Table 1). In Experiments 2 and 3, the body weight of females was significantly lower than males prior to MCAO and two weeks after stroke. This was due to the fact that this study used age-matched male and females.

3.2. Cerebral vascularization in male animals after 90-min MCAO

Representative images of the cortex vasculature were shown in Fig. 1 A. Vascular volume (Fig. 1 B) and surface area (Fig. 1 C) in the cortex area was greater in the diet-enriched diabetic sham group as compared to control rats. By Day 14 after ischemia/reperfusion injury, however, both vascular volume and surface area in the diabetic group decreased to the level of control animals, while there was no change in control animals indicating a disease and intervention (stroke) interaction. This was observed in both ipsilateral and contralateral hemispheres.

Branch density was not significantly increased in the diabetes sham group, but was decreased by Day 14 after ischemia/reperfusion injury, while it was not changed in control animals (Fig. 1 D). Tortuosity had no change before and after ischemia/reperfusion injury in both control and diabetes groups (Fig. 1 E). Similar results were obtained in the striatal regions as well (not shown).

3.3. Functional outcomes in male animals after 90-min MCAO

Neurological function was assessed by multiple tests. Composite score was similar at baseline, but post-stroke recovery was significantly impaired in diabetic group compared to control group (Fig. 2 A). While control rats returned to baseline levels, diabetic rats showed a blunted response. Similar results were obtained with the forelimb grip strength after stroke (Fig. 2 B). Cognition and memory function, evaluated by NOR test (Fig. 2 C), showed lower recognition index in diabetic animals even at the baseline before stroke. In the post-stroke period, recognition index remained lower in the diabetic animals even at 14 days while recovered close to the baseline in control animals.

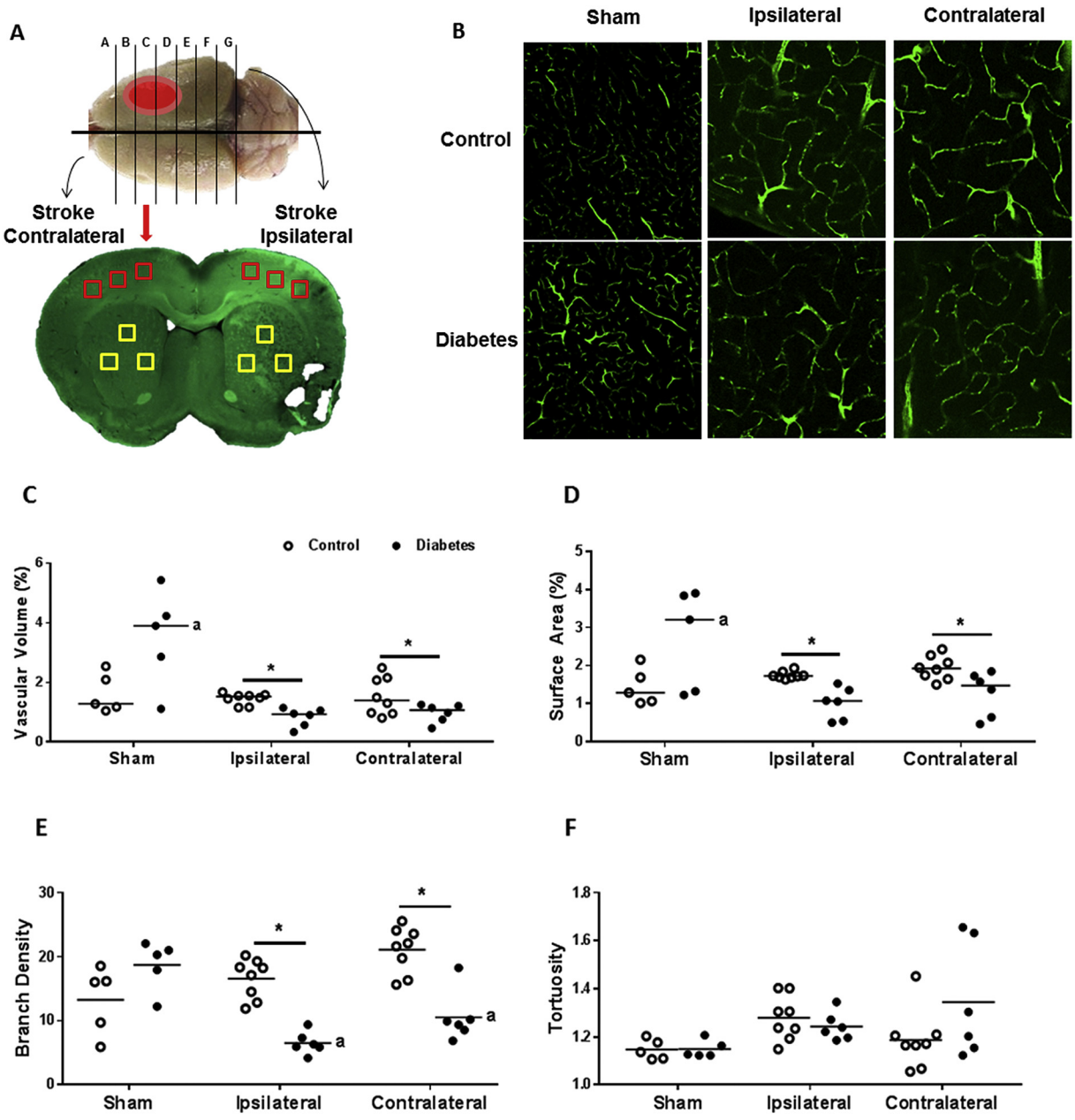

Fig. 1. Effect of diabetes on cerebral vascularization in male animals with 90 min MCAO. A and B, Representative images of FITC-filled vasculature in the cortex of sham or stroked control and diabetes animals are shown. Three regions of interest in cortex (red) or striatum (blue) in section C of the coronal cross-sections, which covers the infarct area (light and heavy pink in the sagittal image) and the mirror images in the contralateral hemisphere, was randomly selected and analyzed. In sham animals, only ipsilateral hemisphere was imaged. While diabetes increases vascular volume (C) and surface area (D) at baseline, vascularization is decreased 14 days after ischemic injury in both hemispheres in diabetes only (C to E), while tortuosity (F) had no significant change. [*$p < 0.01$ for interaction between disease (control vs. diabetes) and surgery (sham vs. stroke); $^{a}p < 0.05$ vs. control]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Cerebral vascularization in male and female animals after 60-min MCAO

The vascularization after 60 min ischemia/reperfusion injury was measured in the ipsilateral side (stroke side) only. By Day 14 after 60 min ischemia/reperfusion injury, vascular volume and surface area measurements indicated no difference between control and diabetic conditions in both male and female groups (Fig. 3 A and B). Tortuosity also had similar pattern (Fig. 3 D). While branch density significantly decreased in the male diabetic group (Fig. 3 C), there was no difference in female control and diabetic groups.

3.5. Motor and cognitive outcomes in male and female animals after 60-min MCAO

Composite scores showed a similar drop in all groups after stroke and by Day 14 all animals restored to baseline levels (Fig. 4 A). Time to remove adhesive tape was variable in the ART. By Day 14, both control and diabetic female groups had recovered close to the level before stroke, while diabetic male animals had the longest removal time (Fig. 4 B). Cognitive deficit was evident in both male and female diabetic animals at baseline before stroke. There was a further decline in both male and female diabetic rats even by Day 14 after 60 min ischemia/reperfusion injury (Fig. 4 C).

Table 1
Metabolic parameters of the animals in each group.

Experiment 1	Male		Female	
	Control (n = 8)	Diabetes (n = 6)	Control	Diabetes
Before MCAO				
Body weight (BW, g)	438 ± 3.4	455 ± 8.1		
Blood glucose (BG, mg/dl)	84 ± 16	230 ± 56**		
14 days post MCAO				
BW (g)	432.7 ± 7.6	449.6 ± 9.36		
BG (mg/dl)	87 ± 16	230 ± 42**		
Hb A1c (%)	5.7 ± 1.1	11.01 ± 0.73**		
Insulin (ng/mL)	4.7 ± 1.5	1.1 ± 0.6*		
Triglycerides (mg/dL)	35.5 ± 5.3	121.0 ± 18.0**		
Cholesterol (mg/dL)	96.6 ± 3.5	179.9 ± 5.8*		
FFA (mmol/L)	0.3 ± 0.03	0.5 ± 0.05*		
Adiposity (% BW)	3.8 ± 0.2	5.2 ± 0.2		
Experiment 2	(n = 7)	(n = 6)	(n = 8)	(n = 6)
Before MCAO				
BW (g)	405 ± 24	374 ± 29	291 ± 27	288 ± 23
BG (mg/dl)	78 ± 10	306 ± 49	75 ± 11	293 ± 44
14 days post MCAO				
BW (g)	443 ± 39	376 ± 40	301 ± 22	269 ± 44
BG (mg/dl)	80 ± 9	288 ± 47	79 ± 7	265 ± 61
Experiment 3	(n = 8)	(n = 6)	(n = 8)	(n = 6)
Before MCAO				
BW (g)	419 ± 24	392 ± 31	307 ± 25	253 ± 31
BG (mg/dl)	74 ± 9	357 ± 83	77 ± 9	272 ± 39
3 days post MCAO				
BW (g)	410 ± 25	318 ± 8	257 ± 34	232 ± 15
BG (mg/dl)	71 ± 6	266 ± 36	72 ± 5	242 ± 42

FFA: free fatty acid.
*$p < 0.05$. **$p < 0.001$ vs. Control.

3.6. Effect of occlusion time on infarct size, edema, and HT

Since there was no difference in functional outcomes at Day 14 post-stroke between control and diabetic animals with the exception of lower recognition index in both diabetic groups, early neurovascular injury was evaluated in Experiment 3 by Day 3 after 60-min MCAO. The first endpoint we measured was infarct size (Fig. 5 A). Male animals had comparable infarct size regardless of disease. In females, infarct size in the control group was smaller than that in male control animals. However, diabetic female rats developed greater infarction compared to their respective control group. Edema percentage was increased in both male and female diabetic groups comparing to the control groups (Fig. 5 B). For the HT measurements, there was no macroscopic bleeding as determined by HT index in female animals, and males had a higher incidence of HT (Fig. 5 C). As shown in Fig. 5 D, the excess Hb content in the ipsilateral side of ischemia/reperfusion injury had similar level in control and diabetes groups, while males had a higher level than the females in both groups, respectively.

4. Discussion

As the search for acute neuroprotective strategies and treatments is still continuing after years of failed trials, significant effort has shifted towards better understanding of the neuroreparative and restorative mechanisms after ischemic brain injury in recent years. Numerous studies conducted using male animals provided evidence that angiogenesis and cerebrovascular remodeling are critical physiological responses for neurovascular repair and comorbid conditions such as diabetes and hypertension can hamper these important repair mechanisms (Abdelsaid et al., 2015; Elgebaly et al., 2011; Kelly-Cobbs et al., 2012). The current study expands this line of research and provides evidence that diet-induced diabetes negates neurovascular protection typically seen in female animals. Furthermore, post-stroke vascularization pattern is influenced by the degree of acute vascular injury and correlates with functional recovery in both sexes.

While diabetes is an endocrine disease due to the lack or insufficiency of insulin action, it is also a vascular disease as most, if not all, complications of the disease have a vascular basis (Forbes and Fotheringham, 2017). Stroke is no exception and it is considered as macrovascular complication for long time due to accelerated atherosclerosis in diabetes. Small vessel disease also contributes to increased risk and severity of stroke in diabetes as evidenced by amplified edema and HT, indicating the vulnerability of diabetes-stricken vessels to a second hit by ischemia/reperfusion injury (Abdelsaid et al., 2015; Ergul et al., 2012). Thus, it is not surprising that numerous clinical and experimental studies have shown that diabetes worsens cognitive and motor outcomes following stroke (Arnold et al., 2014; Capes et al., 2001; Ergul et al., 2012; Ning et al., 2012; Piernik-Yoder and Ketchum, 2013; Prakash et al., 2013b). By the same token, healthy vasculature is also important for the healing process. We have previously reported that there is loss of the cerebrovasculature after stroke in a lean model of spontaneous type 2 diabetes and this was associated with poor functional recovery (Prakash et al., 2013b). Sweetnam et al. showed that in a model of type 1 diabetes, recovery was severely blunted and this was associated with decreased synaptic plasticity (Sweetnam et al., 2012). The same group later showed that branch density is also decreased without a significant change in vascular density (Reeson et al., 2016). Given that 30–40% of ischemic stroke patients have diabetes, diet-induced metabolic derangements are the main cause of the diabetes epidemic and stroke is the leading cause of adult disability, the current study used a clinically relevant and reproducible diet-induced model of diabetes. Several important observations were made. Similar to the GK model of diabetes, vascularization indices were higher in the sham operated diabetic rats than in control animals suggesting that this vascular response is common in different models of diabetes (Prakash et al., 2013a; 2012). Furthermore, there was a decrease in vascularization indices in diabetic animals 14 days after stroke induced by a 90-min MCAO in both hemispheres. The pattern of decreased vascularization was similar to our previous results in GK rats but the magnitude was less in this diet-induced model of diabetes (Prakash et al., 2013b). These results are different from those reported by Ye et al., which showed greater arterial density in the peri-infarct zone in type 1 diabetic mice as compared to nondiabetic mice 14 days after MCAO (Ye et al., 2011). On the other hand, Reeson and colleagues (Reeson et al., 2015) demonstrated there is a reduction in branch points suggesting vascular pruning in type 1 diabetic animals similar to our findings. The greater vasoregression we observed may be due to the differences in the diabetes model used or the extent of the ischemic injury as discussed below.

In the US, there has been a decline in the mortality rate for men with diabetes but not in women (Gregg et al., 2007). This coincides with the report that women with type 2 diabetes have a greater chance of recurrent stroke independent of age, a trend not seen in men (Policardo et al., 2015). Despite the growing evidence that sex plays an important role in the stroke pathophysiology, treatment and outcome, there is still a substantial gap in knowledge in the research field. Therefore, in the next set of experiments we wished to compare functional outcomes and the vascular repair response in male and female animals after a 90-min MCAO as in our first set of experiments. However, due to increased mortality in aged matched diabetic females, we changed our protocol to 60 min MCAO. Surprisingly, there were no difference in the behavioral tests between control and diabetic male and female animals. Furthermore, with the exception of branch density measurements, we did not observe any difference in vascularization indices measured. These results suggest that the severity of the initial injury is a critical factor in

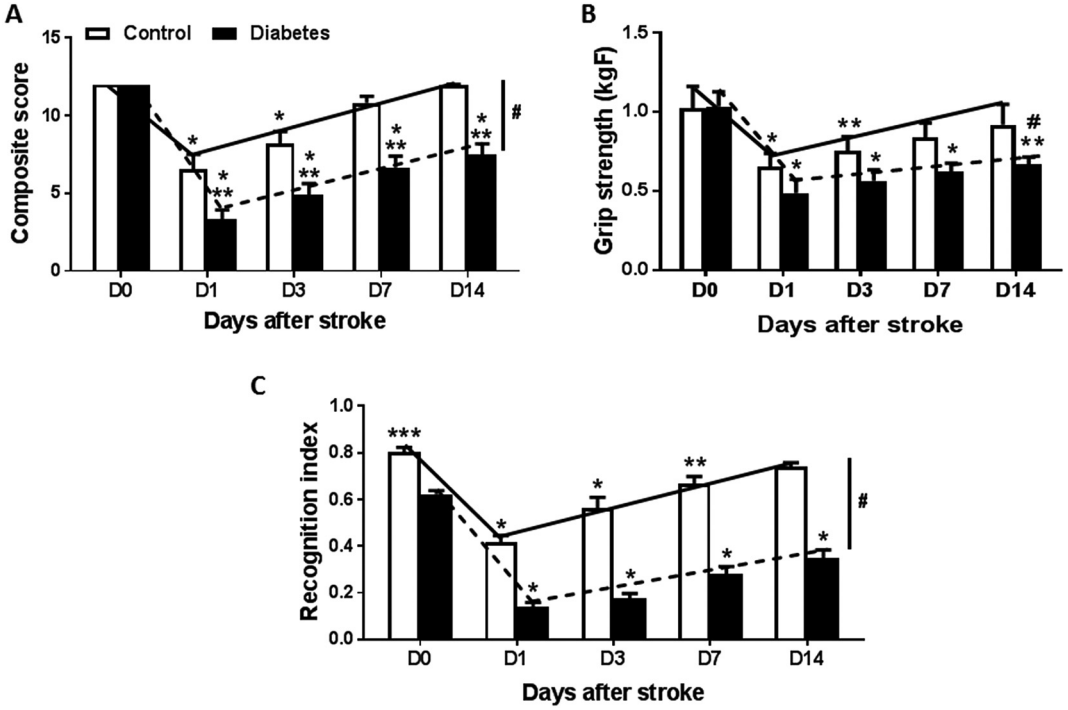

Fig. 2. Diabetes worsens motor and cognitive deficits in male animals with 90 min MCAO. A, Composite score was lower after stroke in the diabetic group and did not return to baseline [*$p < 0.001$ vs. D0, **$p < 0.05$ vs. control, $^{\#}p < 0.01$ for interaction between disease (control vs. diabetes) and days after stroke (D0 through D14)]. B, Diabetes worsened grip strength over the course of 14 days after stroke compared to control. (*$p < 0.001$, **$p < 0.05$ vs. D0; $^{\#}p < 0.05$ vs. control). C, Diabetes had significantly worsened the recognition index for NOR testing by Day 14 after stroke. [*$p < 0.0001$, **$p < 0.05$ vs. D0; ***$p < 0.01$ vs. diabetes; $^{\#}p < 0.01$ for interaction between disease (control vs. diabetes) and days after stroke (D0 through D14)].

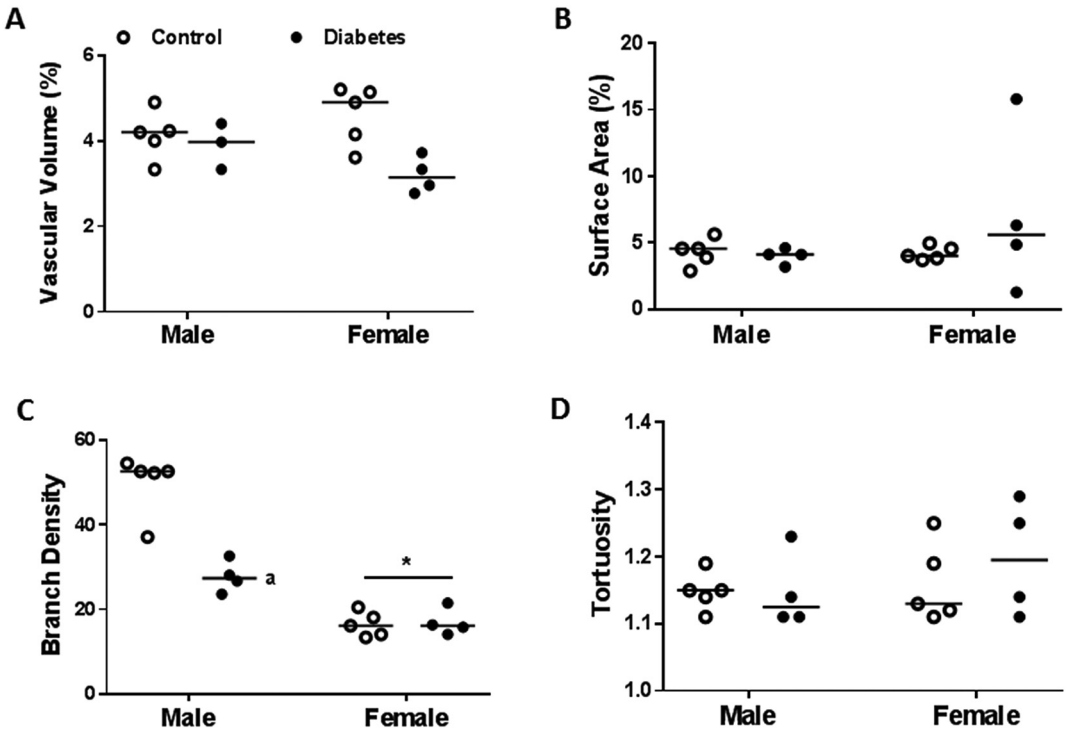

Fig. 3. Effect of diabetes on cerebral vascularization in both male and female rats with 60 min MCAO. There was no difference in vascular volume (A), surface area (B), nor tortuosity (D) regardless of disease or gender. Male diabetic animals had lower branch density (C) than their control counterparts while females exhibited a lower branch density versus males in both control and diabetes animals. [$^{a}p < 0.001$ vs. control, *$p < 0.001$ for interaction between disease (control vs. diabetes) and sex (male vs. female)].

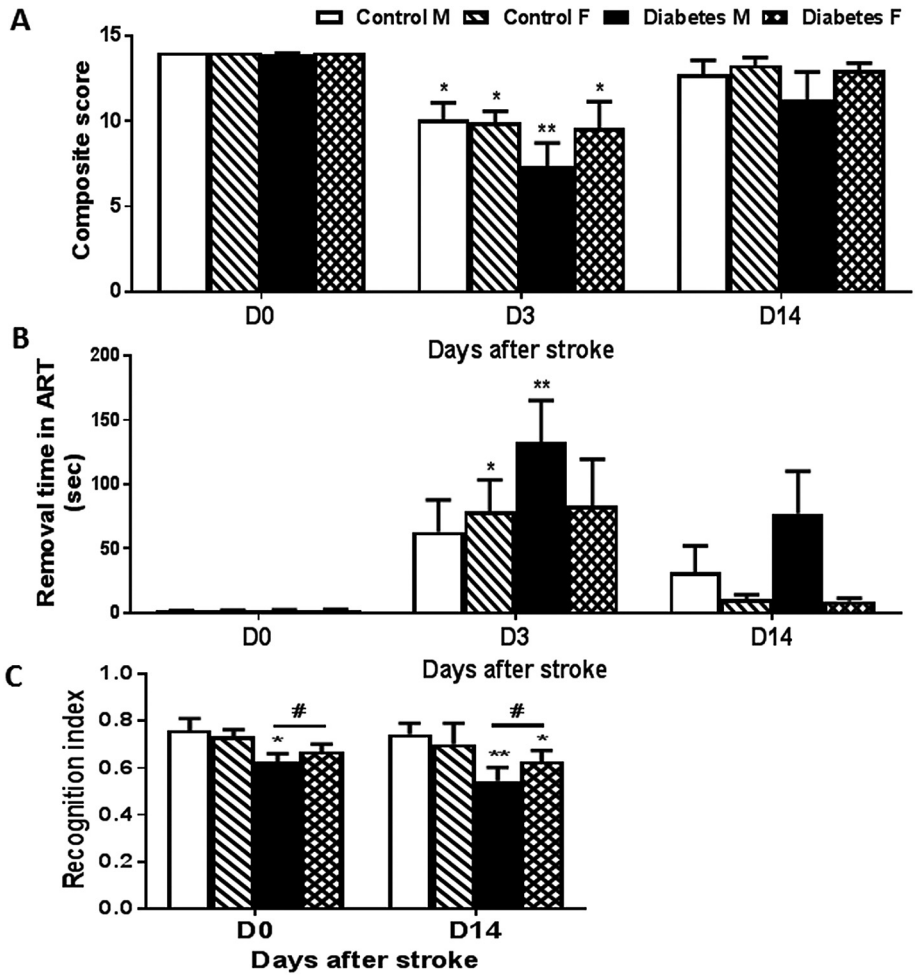

Fig. 4. Sensorimotor and cognitive deficits in male and female rats with 60 min MCAO. A, All groups had exacerbated composite score on Day 3 after stroke and recovered to the baseline at similar level by Day 14 (*$p < 0.01$, **$p < 0.001$ vs. D0). B, Diabetes worsened the removal time of ART in male but not female group (*$p < 0.05$, **$p < 0.01$ vs. D0). C, Diabetes deteriorated cognitive function in both male and female animals by Day 14 after stroke [(*$p < 0.01$, **$p < 0.001$ vs. control; $^{\#}p < 0.05$ interaction between disease (control vs. diabetes) and sex (male vs. female)].

the vascular and functional recovery response. This fact also explained the variable vasoregression findings in type 1 diabetes studies discussed above.

Based on these findings, we compared the early neurovascular injury in the same groups 3 days after 60-min MCAO. First, neuroprotection observed in young female rats was lost when animals were diabetic. One other study reported that ischemic brain damage is much greater in genetically diabetic female KKAy mice as compared to male KKAy mice (Sakata et al., 2011). A possible reason for the loss of neuroprotection in diabetes could be due to the effect of diabetes on estrogen or estrogen receptors. While sexual dimorphism in ischemic stroke cannot be fully attributed to sex hormones, estrogens, especially 17β estradiol (E2), contribute to neuroprotection through activation of estrogen receptor-α (ERα). We did not measure the circulating E2 levels or brain ER expression and distribution in this study. However, when estrus cycle was monitored for stroke surgery timing, there was no notable difference in vaginal swaps between control and diabetic rats. While several clinical studies suggested that diabetes increases E2 levels in postmenopausal women, data on premenopausal women is missing and it is possible that E2 is decreased in our model (Han et al., 2014). ERα in the hypothalamus has been shown to regulate energy expenditure and glucose homeostasis. Interestingly HFD reduces this receptor in male but not female animals (Morselli et al., 2016). Whether ERα expression and distribution are altered in our model remains to be determined. The second important finding was that 60-min MCAO did not cause significant HT. Previous studies reported that 3 h occlusion time that is needed to induce HT in control animals is reduced in diabetic male animals (Li et al., 2013). We recently reported for the first time that 90-min suture or embolic MCAO causes greater bleeding in diabetic female rats (Li et al., 2017). The current study builds upon those findings and show that a shorter occlusion does not cause HT. This finding also raised the possibility that lack of bleeding may be a factor in the lack of vasoregression and differences in functional outcomes as in all our previous studies where we saw these deficits, there was also greater bleeding into the brain.

There are limitations in this study. Due to the increased mortality rate in diabetes, number of animals are less in the diabetic, especially female groups. The mortality in diabetes group was higher than expected when the Exp 1 was finished. Hence, the ischemia time was shortened in both Exp 2 and Exp 3. However, the changes we saw are robust so we believe that this did not affect our conclusions. Second, vascularization indices were measured using a space filling method that requires proper perfusion. Changes in cerebral perfusion in diabetes, especially in the diabetic animals, may influence FITC filling. In previous studies, we confirmed that the decrease in vascularization could be detected by immunohistochemistry for endothelial staining as well. While further studies are needed to determine whether the presence or lack of HT is a determinant of vasoregression and poor recovery observed in diabetes, this translational study provides insight that the severity of the initial injury during ischemia is important for early and

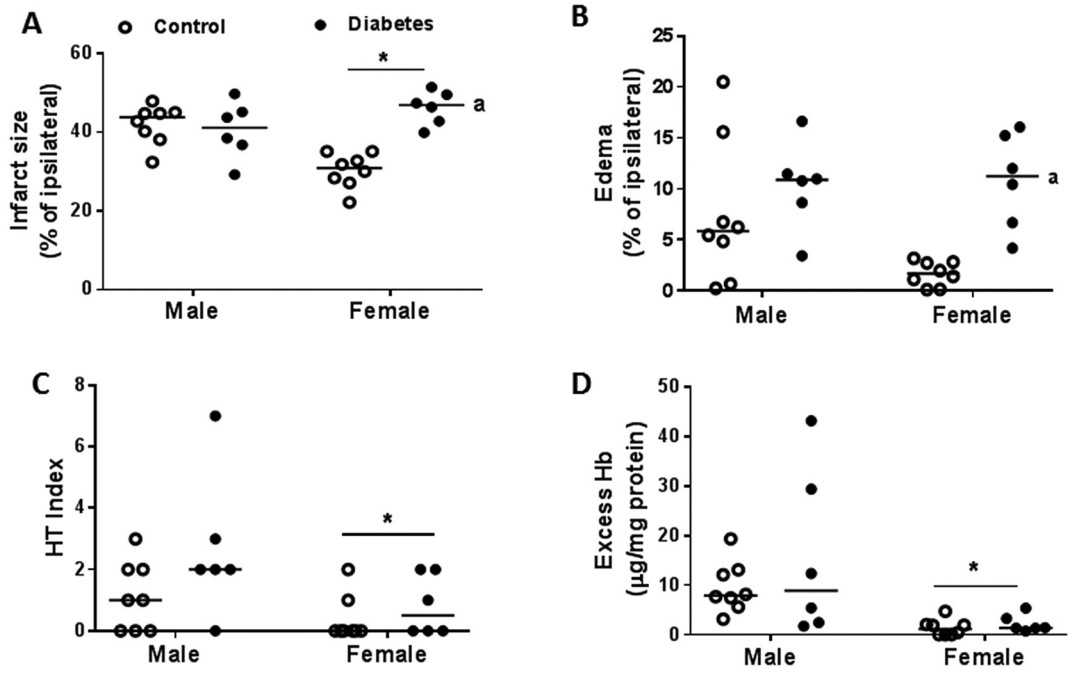

Fig. 5. Diabetes negates neuroprotection in young female rats. A, Infarct size had no change in males but greatly increased in females with diabetes [$^{a}p < 0.001$ vs. control; $^{*}p < 0.001$ for interaction between disease (control vs. diabetes) and sex (male vs. female)]. B, Diabetes increased edema in female but not male animals ($^{a}p < 0.05$ vs. control). C and D, HT determined by macroscopic HT index and excess Hb level was significantly lower in females than their male counterparts, respectively [$^{*}p < 0.05$ for interaction between disease (control vs. diabetes) and sex (male vs. female)].

long-term neurovascular injury and repair. It is also one of the few papers to compare vascular and neuronal injury in male and female control and diabetic animals.

Conflict of interests

All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare. AE is a Senior Research Career Scientist at the Charlie Norwood Veterans Affairs Medical Center in Augusta, GA, USA. The contents do not represent the views of the Department of Veterans Affairs or the US Government.

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