Diabetes Worsens Functional Outcomes in Young Female Rats: Comparison of Stroke Models, Tissue Plasminogen Activator Effects and Sexes

Weiguo Li^{1,2}, Rebecca Ward³, John Paul Valenzuela², Guangkuo Dong^{1,2}, Susan C. Fagan^{1,4}, and Advije Ergul^{1,2,4}

¹Charlie Norwood Veterans Administration Medical Center, Medical College of Georgia, Augusta University, Augusta, GA

²Department of Physiology, Medical College of Georgia, Augusta University, Augusta, GA

³Department of Neuroscience and Regenerative Medicine, Medical College of Georgia, Augusta University, Augusta, GA

⁴Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA

Abstract

Diabetes worsens stroke outcome and increases the risk of hemorrhagic transformation (HT) after ischemic stroke, especially with tissue plasminogen activator (tPA) treatment. The widespread use of tPA is still limited by the fear of hemorrhagic transformation (HT) and underlying mechanisms are actively being pursued in preclinical studies. However, experimental models use a 10 times higher dose of tPA than the clinical dose (10 mg/kg) and mostly employ only male animals. In this translational study, we hypothesized that low dose tPA will improve the functional recovery after the embolic stroke in both control and diabetic male and female animals. Diabetes was induced in age matched male and female Wistar rats with high fat diet and low dose streptozotocin (30 mg/kg, i.p.). Embolic stroke was induced with clot occlusion of the middle cerebral artery (MCA). The animals were treated with or without tPA (1 mg/kg, i.v.) at 90 min after surgery. An additional set of animals were subjected to 90 min MCAO with suture. Neurological deficits (composite score and adhesive removal test-ART), infarct size, edema ratio, and HT index were assessed 3 days after surgery. In the control groups, female rats had smaller infarcts and better functional outcomes. tPA decreased infarct size in both sexes with a greater effect in males. While there was no difference in HT between males and females without tPA, HT was less in the female + tPA

*Address reprint requests to Advije Ergul, M.D., Ph.D., Augusta University, Dept. of Physiology, CA2094, Augusta, GA 30912, Tel: 706-721-9103, Fax: 706-721-7299, aergul@augusta.edu.

Compliance with Ethics Requirements

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

Conflict of Interest

Weiguo Li declares that he has no conflict of interest.

Rebecca Ward declares that she has no conflict of interest.

John Paul Valenzuela declares that he has no conflict of interest.

Guangkuo Dong declares that he has no conflict of interest.

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

Advije Ergul declares that she has no conflict of interest.

group. In the diabetic groups, neuronal injury increased in females reaching that of the infarct sizes seen in male rats. tPA decreased infarct size in females but not males. HT was greater in female rats than in males and was not further increased with tPA. Diabetes worsened neurological deficits in both sexes. Male animals showed improved sensorimotor skills, especially with tPA treatment, but there was no improvement in females. These data suggest that diabetes amplifies neurovascular injury and neurological deficits in both sexes. Human dose tPA offers some degree of protection in male but not female rats. Given that control female animals experience less injury compared to male rats, the diabetes effect is more profound in females.

Introduction

It is well established that diabetes increases the risk and worsens functional outcomes of acute ischemic stroke [1, 2]. Experimental studies using various models of diabetes corroborated the clinical findings and showed that diabetes exacerbates vascular injury, i.e. increased brain swelling, hemorrhagic transformation (HT), and blood brain barrier (BBB) disruption, leading to greater neurological deficits [2–5]. Studies also showed that the extent of neuronal injury varies with blood glucose levels in different models of diabetes. We have reported that there are also differences in the extent of neurovascular injury depending on the stroke model used [6]. Most of these studies employed the middle cerebral artery suture occlusion (MCAO) model of stroke. In a recent study we showed that embolic occlusion of the MCA caused less bleeding compared to the suture occlusion model [6]. While it is known that tissue plasminogen activator (tPA) increases the risk of bleeding to a greater extent in diabetic individuals and tPA remains to be the only FDA-approved therapeutic for ischemic stroke patients, a comparative analysis of neurovascular injury and functional outcome after an embolic stroke in control and diabetic models with and without tPA administration was lacking. Furthermore current embolic stroke models use a 10 times greater dose of tPA than that used in patients, due to the differences in the human and rodent fibrinolytic system [4, 7–10]. Recent studies showed that a lower dose of tPA is also effective in rodents [11]. Thus, the first goal of this translational study was to test the hypothesis that tPA worsens the neurovascular injury and stroke outcomes in diabetes by using the embolic MCAO with or without human dose tPA treatment.

Stroke is a sexually dimorphic disease. It is well established that women are protected from cardiovascular disease including stroke until they reach menopause, after which there is a disproportionate increase in the incidence and severity of ischemic stroke [12, 13]. Given that diabetes increases the stroke risk, especially in younger individuals and females [14], there is an urgent need to better understand stroke recovery in diabetic female subjects. Preclinical studies on diabetic stroke in females are extremely limited, however [15, 16]. Accordingly, the second goal of this study was to test the hypothesis that diabetes exacerbates neurovascular injury and worsens functional outcomes in a sex-independent manner.

Materials and Methods

Study Design and Animal Groups

The animals were housed at the Augusta University animal care facility, which is approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the institutional animal care and use committee. This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research, and adhered to the current RIGOR guidelines for the translational research with respect to 1) *Blinding of the study*; 2) *Randomization of treatment (intervention) groups*; 3) *Power analysis*; and 4) *Statistical analysis* [17, 18]. The animals were randomly selected (in a block size of 2 rats per cage) for the sham, embolic MCAO, suture MCAO, with or without tPA treatment groups. All behavioral testing and data analyses were conducted in a blinded manner.

Wistar male and female rats (Envigo, Indianapolis, IN) at 11–12 weeks of age were used (6–8/group). Diabetes was induced by a 45% high fat diet (HFD, Research Diet Inc, New Brunswick, NJ) starting at 4 weeks of age and a single dose streptozotocin (STZ) injection (30 mg/kg) at 6 weeks of age. Control animals received regular chow. Blood glucose was measured during the light cycle twice per week until stroke surgery and daily after that. Embolic or mechanical suture occlusion was used to induce stroke 6–7 weeks after STZ injection. HFD was continued until sacrifice at Day 3. In a subset of the male and female animals, blood lipid profile and insulin levels were also measured by specific assays as follows: 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/Mouse Insulin ELISA kit from Millipore, (Billerica, MA). Elevated blood glucose levels in diabetic animals were confirmed by hemoglobin (Hb) A1c measurement with Bayer A1cNow system (Fisher Scientific).

Preparation of Blood Clots

The method of clot preparation was adapted from earlier reports and modified to increase clot stability as we recently reported [19, 20]. Briefly, arterial blood from a donor rat was supplemented with human fibrinogen (2 mg/mL) and immediately withdrawn into 20 cm of polyethylene (PE)-50 tubing to clot at room temperature for 6 h and subsequently kept at 4°C for 18 h. The PE-50 tube containing the clot was cut into 5 cm long pieces. The clots were then transferred to a Petri dish containing sterilized saline and left for further retraction at room temperature for 4 h. A single 4 ± 0.5 cm long clot was transferred to a PTFE Sub-lite catheter (Braintree Scientific Inc, Braintree, MA) for surgery.

Stroke Surgery

Focal cerebral ischemia was induced by embolic or suture MCAO as previously described [6, 20]. In Experiment I with embolic stroke, rats were randomly assigned in a block size of 2 rats per cage to 8 different groups: Male or female control vehicle, control tPA, diabetes vehicle and diabetes tPA. In Experiment II with suture MCAO, rats were randomly assigned in a block size of 2 rats per cage to 4 different groups: Male or female control and diabetes. In female rats, estrous cycle was monitored by vaginal swab [21] and stroke surgery was

performed during diestrus phase. Animals were anesthetized with 5% isoflurane, and maintained with 2.0% isoflurane in 70% N₂ and 30% O₂ using a facemask. The PTFE sub-
 lite catheter containing the clot was inserted up to the origin of MCA through the stump of
 external carotid artery (ECA), and the clot was gently injected with 100 µL of the sterile
 saline. The catheter was removed immediately after embolization. Laser Doppler imaging
 with a scanning system (PIM3, Perimed, North Royalton, OH) was used to confirm a
 successful occlusion and ensure similar levels of cerebral blood flow (CBF) reduction in all
 groups 10 min after emboli or suture insertion. Since repeated exposure to isoflurane can
 affect infarct size measurements, in a small set of male animals CBF was monitored over
 time after stroke (Figure 1A and B) to evaluate spontaneous or tPA-mediated resolution of
 the clot. Relative CBF (rCBF) was expressed in the ischemic hemisphere as % of
 nonischemic hemisphere starting with preMCAO measurements. In the embolic model,
 animals received 1 mg/kg tPA (Cathflo Activase (Alteplase), Genentech) or vehicle (sterile
 saline) of equal volume intravenously infused over 20 minutes through the femoral vein
 starting 90 min after cerebral ischemia induction. In the suture model, MCAO duration was
 90 min. Animals were followed up to 72 h after surgery.

Evaluation of Neurovascular Injury

Brain slices (A to G, +6.7 mm to -8.8 mm of bregma) were first imaged for analysis of
 macroscopic bleeding (HT index) and then stained with 2,3,5- triphenyltetrazolium chloride
 (TTC) for infarct size and edema measurements [22]. Infarct size was calculated as the
 percentage of the ischemic hemisphere. Edema was calculated as a percent increase in the
 ischemic hemisphere vs. the contralateral hemisphere. After imaging, the hemispheres were
 separated and deep frozen for tissue hemoglobin quantification with QuantiChrom kit
 (BioAssay Systems, Hayward, CA) and reported as excess hemoglobin (ischemic
 hemisphere - sham Hb, µg/mg protein) [23]. HT index was scored in brain slices B to E
 using a five-point rubric (0 – Ischemic damage with no hemorrhage; 1 – Dispersed
 individual petechiae; 2 – Confluent petechiae; 3 – Small diffuse hemorrhage or hematoma; 4
 – Large diffuse hemorrhage or hematoma) and the total score for each animal was reported
 [24]. All endpoints for neurovascular injury were measured by a blinded investigator.

Behavioral Measurements

Neurobehavioral tests were recorded and scored in a blinded fashion as described before
 [25]. Animals were handled for 5–7 days prior to surgery in rooms where the tests were to
 be carried out. Tests included Bederson score, beam walk, and adhesive removal test (ART)
 before and after stroke at 24 h (Day 1) and 72 h (Day 3). Bederson score for each rat was
 obtained by using 4 parameters which include (a) observation of spontaneous ipsilateral
 circling (2 for no circling, 1 for partial circling and 0 continuous circling), (b) contralateral
 hindlimb and (c) forelimb retraction which measures the ability of the animal to replace the
 limb after it is displaced laterally by 2 to 3 cm (2 for immediate replacement, 1 for
 replacement after minutes, and 0 for no replacement) and (d) resistance to push (1 or 0,
 depending on whether the animal is able to resist pushing or not). Maximum score of 7 was
 allotted to a normal rat. Beam walking ability graded based on a 7-point scale method as
 described by Feeney et al. [26, 27]. Composite neurological score was reported as the sum of
 the Bederson score and the beam walking score on a 0–14 scale with 0 indicating death.

ART was carried out as previously described [28] with modification. Contact and removal latency of the adhesive paper dot was recorded on Day 1 and 3 after MCAO. For each day, the average was taken from 3 trials with a maximum removal latency of 180 seconds per trial.

Data Analysis

Power analysis were made at $\alpha=0.05$. Based on the Hb index data in our preliminary studies (control 1.5 ± 3.0 and diabetic 13 ± 4.0 , mean $\pm$ SD), a sample size of 8/group was predicted to provide at least 85% power to detect the effect of disease and a 25% extra was added due to the increased mortality with tPA treatment.

Mortality was observed either at Day 1 (6–24 h after MCAO) or Day 3 (48–72 h after MCAO). The number of animals that died in each group is shown on Table 1. If an animal died on Day 1, infarct size, edema, Hb or HT results were not included in the analyses since these parameters were measured at Day 3 in most animals. However, since behavioral tests were done at both Days 1 and 3, a composite score of 0 and ART score of 180 sec was included in Day 1 results. If an animal died on Day 3, all endpoints were included in the analyses.

Analysis of change from pre-stroke values for rCBF for control male rats was determined using a paired t-test within time point. The rate of change of rCBF for each rat was determined using linear regression from 10 minutes to 180 minutes post-MCAO. The individual slopes were analyzed in a 2 Disease (control vs. diabetes) X 2 tPA (no vs. yes) ANOVA. Analysis of the effect of diabetes and tPA treatment on infarct size, edema, excess Hb, HT index, and behavioral indexes at Day 1 or Day 3 was performed using a 2 Disease (control vs. diabetes) X 2 tPA (no vs. yes) ANOVA for male and female rats. An interaction between disease and tPA would indicate a differential effect of tPA treatment dependent on disease status. Analysis of the effect of time on functional recovery was performed using a 2 tPA (yes vs no) X 2 Time (Day 1 vs. Day 3) ANOVA for control and diabetic rats. Analysis of the effect of diabetes and sex for infarct size, excess Hb, and behavioral indexes at Day 3 were performed using a 2 Disease (control vs. diabetes) X 2 Sex (male vs. female). An interaction between disease and sex would indicate a differential effect on the outcomes of gender dependent on disease status. A Bonferonni's test was used to determine significant differences among means for significant interactions. SAS 9.3 and GraphPad Prism were used for analyses and statistical significance was determined at $p<0.05$.

Results

Metabolic Profile

Diabetes groups had higher blood glucose levels than control groups in both embolic and suture MCAO studies (Table 1). Body weight was significantly lower in all female groups since groups were age matched to male groups. HFD + low dose STZ intervention also increased plasma lipids in diabetes groups and the increase was greater in male rats (Table 2). Hb A1c level was not significantly different between male and female diabetes animals.

Changes in Cerebral Blood Flow (CBF) Overtime after Embolic Stroke

To determine the temporal profile of spontaneous resolution of the clot, in the first set of experiments, CBF was measured in male rats up to six hours after clot insertion. As shown on Fig 1A, CBF gradually started increasing after 60 min and reached baseline levels by 6 h after surgery. Analysis of slopes from 10 min to 180 min (no tPA 0.12 ± 0.11 vs tPA 0.26 ± 0.14 , $p=0.0041$) showed a treatment effect such that animals receiving human dose tPA showed a faster recovery of CBF regardless of disease status (Fig 1B).

tPA Effects on Neurovascular Injury and Functional Outcomes in Male Rats

Diabetic status did not have an impact on infarct size. While the effect size was greater in control animals, tPA lowered infarct size regardless of the disease status (Fig 2A). Similarly, tPA reduced edema in both groups (Fig 2B). Hemorrhagic transformation (HT) was greater in diabetic rats as indicated by higher Hb values (Fig 2C) and HT index (Fig 2D). tPA treatment increased excess Hb in both control and diabetic groups whereas macroscopic bleeding was increased in the diabetic group.

On Day 1 after stroke, while composite score appeared to be lower in the diabetic group, there was no difference in sensorimotor deficits between groups. Mortality (score of 0) was also similar between control and diabetic groups (Fig 3A). By Day 3, deficits were worsened in vehicle treated diabetic animals but the tPA-treated group showed greater improvement as compared to Day 1 (interaction $p=0.0251$). Mortality (composite score=0) was higher in the diabetic group (Fig 3B). However, due to the wide distribution of scores, there was no significant difference between control and diabetic groups overall. tPA treatment had a similar impact on deficits with a $p=0.07$ indicating a trend for improvement in both groups. ART showed that fine motor skills were lessened in the diabetic group in Day 1 (Fig 3C). Control animals showed a significant improvement especially with tPA treatment by Day 3 but the improvement was very modest in the diabetic rats. There was a trend for higher ART in diabetic animals as compared to control animals at Day 3 ($p=0.07$).

tPA Effects on Neurovascular Injury and Functional Outcomes in Female Rats

While the infarct size was on the higher end of the scale in a majority of diabetic female animals, there was no statistically significant difference between control vs diabetic groups (Fig 4A). tPA had a similar effect in reducing infarction in both groups. For edema, there was a disease and treatment interaction such that tPA reduced edema in the diabetic but not control animals (Fig 4B). Diabetes increased both Hb values and HT index but tPA had no effect on hemorrhage in females (Fig 4C and D). On Day 1 after stroke, composite score was much lower in diabetic animals indicating more deficits. While there was trend for improved outcome in controls treated with tPA ($p=0.09$), it had no effect in the diabetic arm (Fig 5A). By Day 3, control animals recovered to baseline but diabetic animals showed no significant improvement. Mortality (score of 0) was also greater in diabetes (Fig 5B). ART showed that fine motor skills were impaired in the diabetic group in Day 1 (Fig 5C) and tPA had similar effects in both groups. Control animals showed a significant improvement especially with tPA treatment by Day 3 but there was no improvement in the diabetic group (Fig 5D).

The Impact of Sex on Neurovascular Injury Induced by Different Models of Stroke

Comparison of male and female animals subjected to embolic stroke (no tPA) showed that infarct size was smaller in control females but this protection was lost if the female rats had diabetes (Fig 6A). Suture occlusion model showed identical results (Fig 6B). HT was also greater in diabetic animals regardless of the method of stroke and this appeared to be more prominent in female rats (Fig 6C and D). Neurological deficits were lower in female controls as indicated by higher composite scores on Day 3 especially after embolic MCAO. However, this protection was lost in the diabetic setting (Fig 7A and B). ART showed similar results. Control female animals were able to remove the adhesive tape much faster than males but diabetic females failed to do so (Fig 7C and D).

Discussion

The failure of all the therapeutic approaches, with the exception of tPA, in improving outcomes in clinical stroke trials prompted a critical look at the preclinical studies. The Stroke Therapy Academia Industry Roundtable (STAIR) Committee suggested that all promising experimental stroke treatments fail in humans because preclinical studies use relatively young and healthy animals without confounding factors that are commonly found in patients [29]. Given that diabetes, an important comorbidity that is found in almost 30–40% of acute ischemic stroke patients, worsens neurovascular injury and recovery after stroke, there has been a surge in recent years in diabetic stroke research in experimental models. While these past studies made important contributions to our knowledge and identified therapeutic targets to improve recovery in male animals, fundamental questions regarding the impact of sex and use of tPA on stroke outcomes in diabetes remained unanswered. Thus, the current translational study directly addressed these gaps.

Past investigations showed that in all experimental models, diabetes worsened functional outcomes [2, 6, 25, 30–32]. While some studies, including ours, demonstrated that diabetic status does not necessarily increase the infarct size, vascular injury was found to be greater in all diabetic models as compared to control animals [4, 6, 8, 33, 34]. Most of these past studies used the suture occlusion model to induce stroke and studies with the embolic model, especially in combination with tPA, are limited. Fan and colleagues [9] reported that type 1 diabetic male rats are resistant to thrombolytic perfusion resulting in greater infarction and hemorrhage. In a follow up study they reported that early glycemic control reduces injury at 24 h [8] but they did not report functional outcomes. Another group demonstrated that tPA treatment increased hemorrhage and did not reduce infarction or neurological deficits in type 1 diabetic male rats while it was effective in reducing infarct size and improving outcomes in control rats [4]. The current study builds upon these reports and shows that tPA treatment given at 90 min after induction of ischemia effectively reduced infarct size in both female and male control rats but showed a differential effect in diabetes: reducing infarct in females but not males. tPA treatment also reduced the edema, especially in the diabetic animals, which may have resulted from the decrease in infarct size. tPA increased bleeding significantly in both female and male diabetic animals. From a functional point of view, diabetic animals did very poorly. Despite the decrease in infarct size and edema, tPA treatment failed to restore neurological deficits in female and male diabetic animals with the

exception of composite score in male diabetic rats. These findings provide important data with respect to direct comparison of neurovascular injury and neurological function after an embolic stroke with and without tPA treatment in both sexes.

Type 1 diabetes induced by STZ injection is the most commonly used model of diabetes in preclinical stroke studies. Previously, we have used the Goto-Kakizaki (GK) rats, spontaneous type 2 model of diabetes, and showed greater vascular injury after ischemic stroke in both suture and embolic model without significant change in the infarct size [6]. GK rats have mild/moderate hyperglycemia levels around 200 mg/dl. However, this is a lean model with no changes in lipid profile. In addition, female GK rats are resistant to development of diabetes. Among the type 2 diabetic rat models, the HFD plus low dose STZ model was developed more than a decade ago and has been adopted repeatedly in many diabetes focusing on diabetic complications [35–38]. Thus, in the current study we used this model to increase clinical relevance of our studies.

Most, if not all, embolic stroke studies in animal models used approximately 10- fold higher doses of tPA (10 mg/kg) than the dose used in humans (0.9 mg/kg) and this was based on an earlier *in vitro* study which demonstrated that the rodent fibrinolytic system is 10-fold less sensitive to tPA [39]. We and others have shown that low dose tPA (0.9 to 1 mg/kg) is as effective as high dose [7, 11]. In addition to its thrombolytic effects, tPA exerts neurotoxic effects. Thus, in the current study, we used a low dose, close to the human dose, in an embolic model that utilizes a humanized and stable clot [20]. Our data show that, in this model, there is spontaneous resolution of the clot starting within 2 h and CBF returns to baseline levels by 6 h. Administration of tPA accelerates clot lysis in a similar fashion in both control and diabetic groups. This finding is different than an earlier study which reported resistance to tPA-induced reperfusion by 10 mg/kg tPA in a type 1 diabetic rat model [9]. This may be due to differences in the diabetes model and/or the use of a humanized clot that is more amenable to lysis by the recombinant human tPA.

It is well established that women are protected from cardiovascular disease, including stroke, until they reach menopause after which there is a disproportionate increase in the incidence and severity of ischemic stroke [40, 41]. Women suffer from poorer outcomes, greater disability and mortality as well as increased risk of cognitive impairment following stroke [40, 41]. Results from preclinical models using aged or ovariectomized animals suggested that sex hormones, estrogen in particular, may be contributing to the neuroprotection observed in young females and more importantly showed that molecular mechanisms of cell death may differ between males and females emphasizing the importance of studying both sexes in preclinical models [42–45]. Preclinical studies investigating diabetic stroke in females are extremely limited. There is no specific information about HT development in female animals. In the db/db model of type 2 diabetes, it was reported that females develop smaller infarcts than that observed in male mice in a hypoxia model of brain injury [16]. The same group later showed that estrogen stimulates recovery in control but not in diabetic female animals [15]. On the other hand, Sakata et al [14] reported that ischemic injury induced by MCAO is greater in type 2 diabetic female KKAY mice than in male counterparts but there was no difference in neurological deficits. There is clinical evidence that diabetes increases stroke risk, especially in younger individuals and females [46]. Thus, in the current

study, we used aged matched, relatively young, female and male rats. Our results show that, while control females exhibit smaller infarcts than males, as extensively reported in the literature, diabetic female rats develop infarction similar to male animals in both the embolic and suture occlusion models of stroke. Equally important, to the best of our knowledge, this study provides the first evidence of HT, which is greater in female diabetic rats than in male diabetic rats or controls. Results of behavioral tests completely support the neurovascular injury profile with female diabetic animals displaying the greatest deficits.

The goal of this translational study was to compare and contrast the impact of diabetes on stroke outcomes in both sexes. Based on our novel findings, the next step would be ultimately to dissect the underlying mechanisms contributing to the worsened hemorrhage and lack of tPA efficacy in female diabetic rats. While it is purely speculative, it is likely that the reasons are multifactorial and may be due to sex differences in oxidative stress pathways or tPA signaling. It is also known that estrogen activates the coagulation pathways [47] and there may be sex-related effects of diabetes on coagulation pathways and thrombosis. In addition to diabetes, the interaction between tPA signaling and the coagulation pathways in both sexes need to be further studied as well.

In conclusion, this study addressed the complex interaction of diabetes, stroke and tPA in both sexes. We demonstrate, for the first time, that tPA provides neuroprotection in both sexes in control and diabetic conditions. While it offers partial improvement of sensorimotor deficits in male diabetic animals, tPA treatment does not improve outcomes in female diabetic rats. The additional novel findings of this study are that 1) diabetes negates neurovascular protection observed in young female rats, and 2) there is greater HT in female diabetic rats as compared to male rats or controls. As recently reviewed, stroke in women is greatly understudied [45]. Our findings strongly support the notion that including both sexes with comorbid conditions is especially important in both preclinical and clinical studies that evaluate potential stroke therapies.

Acknowledgments

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

References

1. Baird TA, et al. Persistent poststroke hyperglycemia is independently associated with infarct expansion and worse clinical outcome. *Stroke*. 2003; 34(9):2208–14. [PubMed: 12893952]
2. Ergul A, et al. Cerebrovascular Complications of Diabetes: Focus on Stroke. *Endocr Metab Immune Disord Drug Targets*. 2012; 12(2):148–158. [PubMed: 22236022]
3. Yan T, et al. Neurorestorative Therapy of Stroke in Type 2 Diabetes Mellitus Rats Treated With Human Umbilical Cord Blood Cells. *Stroke*. 2015; 46(9):2599–606. [PubMed: 26243222]
4. Ning R, et al. Tissue plasminogen activator treatment of stroke in type-1 diabetes rats. *Neuroscience*. 2012; 222:326–32. [PubMed: 22820263]
5. Ye X, et al. Niaspan reduces high-mobility group box 1/receptor for advanced glycation endproducts after stroke in type-1 diabetic rats. *Neuroscience*. 2011; 190:339–45. [PubMed: 21683770]

6. Li W, et al. Comparative analysis of the neurovascular injury and functional outcomes in experimental stroke models in diabetic Goto-Kakizaki rats. *Brain Res.* 2013; 1541:106–14. [PubMed: 24144674]
7. Hafez S, et al. Comparative Analysis of Different Methods of Ischemia/Reperfusion in Hyperglycemic Stroke Outcomes: Interaction with tPA. *Transl Stroke Res.* 2015; 6(3):171–80. [PubMed: 25683354]
8. Fan X, et al. Early insulin glycemic control combined with tPA thrombolysis reduces acute brain tissue damages in a focal embolic stroke model of diabetic rats. *Stroke.* 2013; 44(1):255–9. [PubMed: 23117722]
9. Fan X, et al. A rat model of studying tissue-type plasminogen activator thrombolysis in ischemic stroke with diabetes. *Stroke.* 2012; 43(2):567–70. [PubMed: 22052516]
10. Won SJ, et al. Hyperglycemia promotes tissue plasminogen activator-induced hemorrhage by Increasing superoxide production. *Ann Neurol.* 2011; 70(4):583–90. [PubMed: 22002675]
11. Haelewyn B, Risso JJ, Abiraini JH. Human recombinant tissue- plasminogen activator (alteplase): why not use the ‘human’ dose for stroke studies in rats? *J Cereb Blood Flow Metab.* 2010; 30(5): 900–3. [PubMed: 20216551]
12. Manwani B, et al. Sex differences in ischemic stroke sensitivity are influenced by gonadal hormones, not by sex chromosome complement. *J Cereb Blood Flow Metab.* 2015; 35(2):221–9. [PubMed: 25388681]
13. Ritzel RM, Capozzi LA, McCullough LD. Sex, stroke, and inflammation: the potential for estrogen-mediated immunoprotection in stroke. *Horm Behav.* 2013; 63(2):238–53. [PubMed: 22561337]
14. Bejot Y, Giroud M. Stroke in diabetic patients. *Diabetes Metab.* 2010; 36(Suppl 3):S84–7. [PubMed: 21211741]
15. Zhang L, et al. Estrogen stimulates microglia and brain recovery from hypoxia- ischemia in normoglycemic but not diabetic female mice. *J Clin Invest.* 2004; 113(1):85–95. [PubMed: 14702112]
16. Vannucci SJ, et al. Experimental stroke in the female diabetic, db/db, mouse. *J Cereb Blood Flow Metab.* 2001; 21(1):52–60. [PubMed: 11149668]
17. Lapchak PA, Zhang JH, Noble-Haeusslein LJ. RIGOR guidelines: escalating STAIR and STEPS for effective translational research. *Transl Stroke Res.* 2013; 4(3):279–85. [PubMed: 23658596]
18. Landis SC, et al. A call for transparent reporting to optimize the predictive value of preclinical research. *Nature.* 2012; 490(7419):187–91. [PubMed: 23060188]
19. Zhang RL, et al. A rat model of focal embolic cerebral ischemia. *Brain Res.* 1997; 766(1–2):83–92. [PubMed: 9359590]
20. Hoda MN, et al. Sex-independent neuroprotection with minocycline after experimental thromboembolic stroke. *Exp Transl Stroke Med.* 2011; 3(1):16. [PubMed: 22177314]
21. Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res B Dev Reprod Toxicol.* 2007; 80(2):84–97. [PubMed: 17342777]
22. Ergul A, et al. Increased hemorrhagic transformation and altered infarct size and localization after experimental stroke in a rat model type 2 diabetes. *BMC Neurol.* 2007; 7:33. [PubMed: 17937795]
23. Qin Z, et al. Hyperbaric oxygen-induced attenuation of hemorrhagic transformation after experimental focal transient cerebral ischemia. *Stroke.* 2007; 38(4):1362–7. [PubMed: 17322079]
24. Kelly-Cobbs AI, et al. Targets of vascular protection in acute ischemic stroke differ in type 2 diabetes. *Am J Physiol Heart Circ Physiol.* 2013; 304(6):H806–15. [PubMed: 23335797]
25. Prakash R, et al. Vascularization pattern after ischemic stroke is different in control versus diabetic rats: relevance to stroke recovery. *Stroke.* 2013; 44(10):2875–82. [PubMed: 23920018]
26. Feeney DM, Gonzalez A, Law WA. Amphetamine, haloperidol, and experience interact to affect rate of recovery after motor cortex injury. *Science.* 1982; 217(4562):855–7. [PubMed: 7100929]
27. Kelly-Cobbs AI, et al. Targets of Vascular Protection in Acute Ischemic Stroke Differ in Type 2 Diabetes. *Am J Physiol Heart Circ Physiol.* 2013; 304(6):806–815.

28. Chen J, Li Y, Chopp M. Intracerebral transplantation of bone marrow with BDNF after MCAo in rat. *Neuropharmacology*. 2000; 39(5):711–6. [PubMed: 10699437]
29. Fisher M, et al. Update of the stroke therapy academic industry roundtable preclinical recommendations. *Stroke*. 2009; 40(6):2244–50. [PubMed: 19246690]
30. Yan T, et al. Niaspan increases axonal remodeling after stroke in type 1 diabetes rats. *Neurobiol Dis*. 2012; 46(1):157–64. [PubMed: 22266016]
31. Li Q, et al. Deficient eNOS phosphorylation is a mechanism for diabetic vascular dysfunction contributing to increased stroke size. *Stroke*. 2013; 44(11):3183–8. [PubMed: 23988642]
32. Chen J, et al. Adverse effects of bone marrow stromal cell treatment of stroke in diabetic rats. *Stroke*. 2011; 42(12):3551–8. [PubMed: 21940967]
33. Elgebaly MM, et al. Vascular protection in diabetic stroke: role of matrix metalloprotease-dependent vascular remodeling. *J Cereb Blood Flow Metab*. 2010; 30(12):1928–38. [PubMed: 20664613]
34. Ding G, et al. Persistent cerebrovascular damage after stroke in type two diabetic rats measured by magnetic resonance imaging. *Stroke*. 2015; 46(2):507–12. [PubMed: 25523056]
35. Reed MJ, et al. A new rat model of type 2 diabetes: the fat-fed, streptozotocin- treated rat. *Metabolism*. 2000; 49(11):1390–4. [PubMed: 11092499]
36. Ding SY, et al. Pioglitazone can ameliorate insulin resistance in low-dose streptozotocin and high sucrose-fat diet induced obese rats. *Acta Pharmacol Sin*. 2005; 26(5):575–80. [PubMed: 15842776]
37. Zhang M, et al. The characterization of high-fat diet and multiple low-dose streptozotocin induced type 2 diabetes rat model. *Exp Diabetes Res*. 2008; 2008:704045. [PubMed: 19132099]
38. Wang C, et al. Ameliorative effect of berberine on endothelial dysfunction in diabetic rats induced by high-fat diet and streptozotocin. *Eur J Pharmacol*. 2009; 620(1–3):131–7. [PubMed: 19686728]
39. Korninger C, Collen D. Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various animal species in vitro. *Thromb Haemost*. 1981; 46(2):561–5. [PubMed: 6795744]
40. Bushnell CD, et al. Advancing the study of stroke in women: summary and recommendations for future research from an NINDS-Sponsored Multidisciplinary Working Group. *Stroke*. 2006; 37(9):2387–99. [PubMed: 16857945]
41. Howe MD, McCullough LD. Prevention and management of stroke in women. *Expert Rev Cardiovasc Ther*. 2015; 13(4):403–15. [PubMed: 25747877]
42. Manwani B, et al. Functional recovery in aging mice after experimental stroke. *Brain Behav Immun*. 2011; 25(8):1689–700. [PubMed: 21756996]
43. Manwani B, McCullough LD. Sexual dimorphism in ischemic stroke: lessons from the laboratory. *Womens Health (Lond Engl)*. 2011; 7(3):319–39. [PubMed: 21612353]
44. Liu M, et al. Mechanisms of gender-linked ischemic brain injury. *Restor Neurol Neurosci*. 2009; 27(3):163–79. [PubMed: 19531872]
45. Ahnstedt H, McCullough LD, Cipolla MJ. The Importance of Considering Sex Differences in Translational Stroke Research. *Transl Stroke Res*. 2016; 7(4):261–73. [PubMed: 26830778]
46. Writing Group, M., et al. Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation*. 2016; 133(4):e38–360. [PubMed: 26673558]
47. Campbell SJ, et al. Contact factor mediated fibrinolysis is increased by the combined oral contraceptive pill. *Br J Obstet Gynaecol*. 1993; 100(1):79–84. [PubMed: 8427844]

Figure 1.

Relative cerebral blood flow (rCBF, % of pre MCAO) change over time after emboli insertion. A. Spontaneous resolution of the clot started 2 h after the occlusion and gradually recovered to baseline levels by 6 h after ischemia in control male rats. Individual data points from 6 animals are shown by symbols and the average change is indicated by the line graph (^a $p < 0.001$ vs pre, ^b $p < 0.01$ vs pre). B. tPA treatment given at 90 min after occlusion over a 20 min period significantly increased the blood flow compared to the vehicle-treated animals, while there was no difference between control and diabetes groups (^a $p < 0.01$ vs vehicle). $n = 6/\text{group}$.

Figure 2.

Effects of tPA treatment on neurovascular injury in male control and diabetic rats. tPA decreased infarct size (A) and edema (B) while increasing excess Hb levels (C) in both control and diabetes rats. Macroscopic bleeding (D, HT index) was increased in diabetic but not control rats (^a $p < 0.05$ vs vehicle, ^a $p < 0.01$ vs vehicle, ^c $p < 0.0001$ vs vehicle). Data is expressed as a scatter plot and the median is indicated by a blue (vehicle) or red (tPA) line. $n = 5-7$ /group.

Figure 3.

Effects of tPA treatment on functional outcomes in male control and diabetic rats. A. There was no difference in composite score on Day 1 after MCAO in all groups. B. By Day 3, the vehicle diabetic group had worse composite scores and higher mortality (0 scores), while tPA treated diabetic animals showed improvement compared to Day 1 (interaction $b_p=0.0251$ vs Day 1). There was a trend for tPA-mediated improvement of neurological deficits in both control and diabetes groups ($a_p=0.077$ vs vehicle). C. ART time was higher in the diabetic group regardless of tPA treatment on Day 1 ($a_p<0.05$ vs control). D. tPA improved fine motor skills as indicated by better ART time in control rats by Day 3 as compared to Day 1 ($a_p<0.05$ vs Day 1, $b_p=0.07$ vs control). Data is expressed as a scatter plot and the median is indicated by a blue (vehicle) or red (tPA) line. $n=7-8$ /group Day 1, $5-7$ /group Day 3.

Female

Figure 4.

Effects of tPA treatment on neurovascular injury in female control and diabetic rats. A. There was no difference in infarct size between control and diabetic groups. tPA reduced neuronal injury (^a $p < 0.05$ vs vehicle). B. Diabetes increased edema and tPA was effective in reducing edema in diabetic but not control rats (interaction, ^a $p = 0.017$). C. Control animals had very low excess Hb but diabetes increased hemorrhage (^a $p < 0.01$ vs control). D. HT index was higher in diabetic animals with or without tPA treatment (^a $p < 0.001$ vs control). Data is expressed as a scatter plot and the median is indicated by a green (vehicle) or pink (tPA) line. $n = 5-8$ /group.

Female

Figure 5.

Effects of tPA treatment on functional outcomes in female control and diabetic rats. A. Composite score was lower in the diabetes group on Day 1. tPA treatment had no effect on deficits in diabetic rats (^a $p < 0.05$ vs control). B. tPA treatment significantly improved the composite score on Day 3 in control rats, but not in the diabetes group (^a $p < 0.0002$ vs control). C. Majority of the diabetic animals could not remove the adhesive tape in 180 sec time frame. tPA treatment had no effects on Day 1 (^a $p < 0.05$ vs control). D. While control animals significantly improved by Day 3, especially with tPA treatment (^b $p < 0.01$ vs Day 1), diabetic animals still did very poorly (^a $p < 0.002$ vs control). Data is expressed as a scatter plot and the median is indicated by a green (vehicle) or pink (tPA) line. $n = 8-10$ /group Day 1, $5-8$ /group Day 3.

Figure 6.

Neurovascular injury in male and female rats with different stroke models. Female control rats had smaller infarct size compared to male rats in both embolic (A) and suture (B) models while diabetes abolished this protection (^a $p < 0.05$ vs male, ^b $p < 0.05$ vs control). Both male and female diabetic rats had higher excess Hb level after embolic (C) or suture MCAO (D) (^a $p < 0.001$ vs control). $n = 5-8$ /group in embolic MCAO and $6-8$ /group in suture MCAO.

Figure 7.

Functional outcomes in male and female rats with different stroke models. Female control rats had higher (better) composite score on Day 3 after embolic (A) and suture (B) MCAO and diabetes negated this protection (^b $p < 0.05$ vs male, ^a $p < 0.001$ vs control). Control female rats had a trend for shorter ART time on Day 3 after embolic (^b $p = 0.1$ vs male) but not suture MCAO. Both female and male diabetic animals did very poorly in ART with most animals not being able to remove the tape in the allowed 180 sec time frame (C and D) (^a $p < 0.05$ vs control, ^c $p < 0.001$ vs control). $n = 5-8$ /group in embolic MCAO and $6-8$ /group in suture MCAO.

Table 1

Physiological parameters of study groups

Sex	Male			Female		
Disease	Diabetes			Diabetes		
Surgery	Control			Control		
Treatment	Embolic MCAO			Embolic MCAO		
BW (g)	Vehicle	tPA	Vehicle	tPA	Vehicle	tPA
BG ^a (mg/dL)	325.7±4.7	316.3±4.5	321.4±22.7	363.0±5.0	270.2±3.9 [#]	262.8±3.7 [#]
BG ^b (mg/dL)	85.1±2.3	77.8±2.9	291.1±18.7 [*]	272.9±10.9 [*]	79.6±3.7	74.9±1.5
BG ^b (mg/dL)	76.6±2.6	75.6±4.0	227.3±16.7 [*]	262.2±20.0 [*]	75.4±2.4	74.2±3.8
Mortality	2/8	2/9	4/9	4/10	0/8	4/9
Surgery	Suture MCAO			Suture MCAO		
BW (g)	Vehicle	tPA	Vehicle	tPA	Vehicle	tPA
BG ^a (mg/dL)	313.9±3.3	333.8±4.9	248.1±4.7 [#]	252.2±9.8 [#]	268.0±18.6 [*]	210.6±28.3 [*]
BG ^b (mg/dL)	76.0±2.6	307.4±19.4 [*]	73.6±2.4	70.1±2.9	3/9	6/10
Mortality	0/9	4/9	1/9			

BW: body weight; BG^a: blood glucose level at MCAO surgery; BG^b: blood glucose level at sacrifice. Mortality are animal numbers of dead/total.

* p<0.001 vs. Ctrl,

[#] p<0.001 vs. male.

Table 2

Metabolic parameters in the HFD + STZ model of diabetes

Sex	Male		Female	
Disease	Control	Diabetes	Control	Diabetes
Insulin (ng/mL)	1.8±0.5	2.8±1.4	1.7±0.5	1.7±1.3
Triglycerides (mg/dL)	35.5±5.3	121.0±18.0 **	42.5±7.5	87.7±23.0 **
Cholesterol (mg/dL)	61.9±4.8	105.0±19.3 *	51.3±3.8	60.7±5.9 *
FFA (μmol/L)	0.3±0.03	0.5±0.05 *	0.23±0.03	0.32±0.04 *
Hb A1c (%)	–	11.01±0.73	–	11.44±0.37

FFA: free fatty acid.

*
p<0.05,**
p<0.0001 vs. Control.