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Comparative analysis of the neurovascular injury and functional outcomes in experimental stroke models in diabetic Goto-Kakizaki rats

Weiguo Li^{a,b}, Zhi Qu^b, Roshini Prakash^c, Connie Chung^b, Handong Ma^{a,b}, Md Nasrul Hoda^d, Susan C Fagan^{a,c}, and Advije Ergul^{a,b,c}

^aCharlie Norwood Veterans Affairs Medical Center, Augusta, GA, USA 30904

^bDepartment of Physiology, Medical College of Georgia, Georgia Regents University, Augusta, GA, USA 30912

^cProgram in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA, USA 30912

^dDepartment of Medical Laboratory, Imaging & Radiologic Sciences, and Neurology, Georgia Regents University, Augusta, GA, USA 30912

Abstract

Diabetes worsens functional outcome and is associated with greater hemorrhagic transformation (HT) after ischemic stroke. We have shown that diabetic Goto-Kakizaki (GK) rats develop greater HT and neurological deficit despite smaller infarcts after transient middle cerebral artery occlusion (MCAO) with the suture model. However, the impact of 1) the duration of ischemia/reperfusion (I/R); 2) the method of ischemia; and 3) acute glycemic control on neurovascular injury and functional outcome in diabetic stroke remained unanswered. Wistar and GK rats were subjected to variable MCAO by suture or embolus occlusion. A group of GK rats were treated with insulin or metformin before stroke with suture occlusion. In all groups, infarct size, edema, HT occurrence and severity, and functional outcome were measured. Infarct size at 24 h was smaller in GK rats with both suture and embolic MCAO, but expanded with longer reperfusion period. Edema and HT were increased in GK rats after 90 min and 3 h occlusion with the suture model, but not in the embolic MCAO. Neurological deficit was greater in diabetic rats. These findings suggest that diabetes accelerates the development of HT and amplifies vascular damage in the suture model where blood flow is rapidly reestablished. Acute metformin treatment worsened the infarct size, HT, and behavior outcome, whereas insulin treatment showed a protective effect. These results suggest that the impact of ischemia/reperfusion on neurovascular injury and functional outcome especially in disease models needs to be fully characterized using different models of stroke to model the human condition.

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Corresponding author: Advije Ergul, MD, PhD, Professor, Department of Physiology, Medical College of Georgia, Georgia Regents University, 1120 15th Street, CA 2094, Augusta, GA, USA 30912, Tel: 1-706-721-9103, Fax: 1-706-721-7299, aergul@gru.edu.

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Keywords

Ischemic stroke; diabetes; infarct; edema; hemorrhagic transformation; middle cerebral artery occlusion

1. Introduction

Diabetes is an increasingly growing epidemic affecting 21 million Americans and over 65% of whom will eventually die of a cardiovascular event such as stroke (Goldstein et al., 2006; Kravetz and Federman, 2009; Lloyd-Jones et al., 2009; Turnbull et al., 2009; Weiss et al., 2009). Since diabetic patients are at a higher risk of stroke and have poorer prognosis compared to the non-diabetic population, a better understanding of the effects of diabetes on ischemic stroke outcome is pivotal for developing better prevention and treatment strategies before and after an ischemic insult (Folsom et al., 1999; Poppe et al., 2009; Stegmayr and Asplund, 1995). Previously we reported that diabetic Goto-Kakizaki (GK) rats develop greater hemorrhagic transformation (HT) and poorer functional outcome despite smaller infarcts 24 h after stroke induced by middle cerebral artery occlusion (MCAO) with 3 h ischemia and 21 h reperfusion (Elgebaly et al., 2010; Ergul et al., 2007). Generated from glucose intolerant Wistar rats, the GK rat is a non-obese model of spontaneous type 2 diabetes with moderately elevated glucose levels (Farese et al., 1994; Standaert et al., 2004). This model provided the possibility to study the effect of hyperglycemia on neurovascular injury and outcome without the influences of comorbidity like hypertension and obesity.

Translational research using different models of experimental ischemic stroke provides indispensable insight not only for the biology of the ischemic stroke, but also for the discovery of novel treatments for ischemic stroke and is highly recommended by the Stroke Therapy Academia Industry Roundtable (STAIR) Committee (Fisher et al., 2009). Ischemic stroke is the most frequent type of stroke, accounting for over 80% of all strokes, in which the middle cerebral artery (MCA) territory is mostly affected (Gillum, 2002). Thus, in this study using suture and thromboembolic occlusion of MCA with different durations of ischemic and reperfusion (I/R) and multiple approaches to regulate blood glucose, we tested the hypotheses that: 1) regardless of the method of I/R, diabetes worsens stroke outcome, and 2) acute glycemic control lessens neurovascular injury and improves outcome in diabetes.

2. Results

2.1. Physiological parameters

GK rats weigh slightly less than controls at the same age but there was no difference in body weight across study groups (Table 1). Blood glucose was higher in diabetic GK rats compared to Wistar (Wis) rats and both metformin and insulin normalized blood glucose in GK rats (Table 2).

2.2. Neurovascular injury and outcome in different models of stroke

The infarct size at 24 h was significantly lower and mainly subcortical in diabetic GK rats as compared to control animals irrespective of the duration and method of occlusion, whereas at 7 days infarct size expanded to the cortex in GK rats (GK 3–7) and was comparable to control animals (Wis 3–7) (Fig 1A and B). The degree of decrease in cerebral blood flow after occlusion was similar among groups (Fig 1C). Edema and hemorrhagic transformation were measured as indices of vascular injury at 24 h. Edema ratio was greater in diabetic animals than in control animals when I/R was achieved by the suture occlusion model (Fig 2A and B, GK 3–21 and GK 90–23 vs. Wis, respectively).

Both measures of HT, HT index and tissue Hb, exhibited a similar pattern. HT index and excess Hb in the ischemic hemisphere were greater in diabetic animals than those measured in control groups (Fig 2C and D, GK 3–21 and GK 90–23 vs. Wis, respectively).

Sensorimotor function after stroke was evaluated by grip strength and a composite neurological deficit score both of which was lower in diabetic animals indicating poor outcome at 24 h and 7 days (Fig 3A and B, each group of GK vs. Wis).

2.3. Glycemic control and neurovascular injury in diabetes

GK strain of rats that have normal blood glucose levels developed greater infarction compared to diabetic GK rats. Glycemic control by insulin (GK ins) did not have an effect on infarct size but metformin treatment (GK met) significantly increased infarction (Fig 4A and B).

Edema ration was lower in normoglycemic GK rats (Fig 4C, GK NBG) and the HT index was lower in insulin treatment group (Fig 5A and B, GK ins vs. GK). Metformin augmented the severity of bleeding as indicated by greater tissue Hb levels (Fig 5C, GK met vs. GK).

Metformin treatment decreased the grip strength (GK met vs. GK). Insulin treatment improved grip strength but had no effect on composite deficit score (GK ins vs. GK). On the other hand, GK rats with normal blood glucose levels (GK NBG vs. GK) displayed a better outcome in both measurements (Fig 6A and B).

3. Discussion

Clinical and experimental studies have shown that diabetes increases the risk and severity of stroke ultimately resulting in poor outcomes (Ennis and Keep, 2007; Knoflach et al., 2012). However, most if not all preclinical evidence came from studies in which suture occlusion of MCA was used to induce stroke (Ergul et al., 2012) and long-term outcome data are limited. Furthermore, whether glycemic control at the time of stroke in previously untreated diabetic subjects reduces injury and improves outcome is not known. Therefore, this study addressed these important questions using a novel humanized thromboembolic clot model as well as intraluminal suture occlusion of MCA with different duration of ischemic and reperfusion (I/R) periods and multiple approaches to regulate blood glucose.

The STAIR Committee (Fisher et al., 2009) suggested that some of the reasons why all promising experimental stroke treatments failed in humans include the use of relatively young and healthy animals without confounding factors that are commonly found in patients, lack of comparative studies on multiple models of stroke, and lack of long-term functional information. Diabetes being a very important comorbidity in acute ischemic stroke patients, preclinical studies followed STAIR recommendations and in last several years studies involving diabetic stroke exponentially increased as recently reviewed (Ergul et al., 2012). However, with the exception of few studies (Fan et al., 2012; Fan et al., 2013; Ning et al., 2012), most studies used temporary focal ischemia induced by the intraluminal suture occlusion of MCA in control versus type 1 diabetic rats. Using this stroke model and the lean GK model of type 2 diabetes, in a series of studies we showed that type 2 diabetes mediates pathological remodeling and neovascularization of the brain, and I/R injury superimposed on this preexisting vascular disease causes extensive vascular injury and bleeding into the brain (Elewa et al., 2009; Elgebaly et al., 2010; Ergul et al., 2007; Kelly-Cobbs et al., 2013). Despite the fact that GK rats developed relatively smaller infarcts compared to control animals at 24 h after stroke, the functional outcome was worse most likely due to increased bleeding and edema. These studies underscored the importance of studying stroke in disease models as recommended by the STAIR Committee.

The current study builds upon these findings and provides important evidence with regard to the impact of duration and method of occlusion and reperfusion on neurovascular injury in diabetes. First is the importance of the method of reperfusion on vascular injury in diabetes. Both experimental and clinical studies suggest that diabetes increases edema and bleeding into the brain after stroke (Ergul et al., 2012). Intracerebral HT is a complex phenomenon that frequently accompanies ischemic stroke, mainly because of disruptions of the blood-brain barrier (BBB) (Lapchak, 2002; Lyden and Zivin, 1993). This devastating phenomenon can occur spontaneously or can be triggered after reperfusion, which is mostly seen in the acute ischemic stroke patients with tissue plasminogen activator (tPA) treatment. Our current findings show that vascular injury indices, HT and edema, are increased in diabetic animals subjected to suture but not thromboembolic stroke. One potential explanation is that in the suture model where reperfusion is achieved abruptly due to filament withdrawal, as opposed to gradual return of blood flow via spontaneous clot lysis in thromboembolic stroke, reperfusion injury caused by the oxidative burst is greater and the fragile vessels of diabetic animals are more vulnerable to this insult. Obviously, embolic model recapitulates the clinical scenario where the clot persists with slow spontaneous reperfusion. The model also prevents the rapid evolution of the penumbra. Given the clinical evidence that tPA increases the risk of bleeding in diabetic patients, this important finding needs to be further expanded and reevaluated using tPA in this model.

The impact of the duration of occlusion on HT development is another important outcome of the current study. A previous study showed that 50% of control animals subjected to focal temporary brain ischemia develop HT if occlusion is 3 h or longer (Fagan and Garcia, 1999). In the current study, even a 90 min occlusion caused significant bleeding in diabetic animals suggesting that preexisting vascular disease renders the vasculature extremely sensitive of oxidative injury and accelerates HT development.

A third important finding of the current study is the importance of duration of reperfusion on infarct development in diabetes. While infarct size was relatively smaller and displayed a subcortical localization at 24 h with both suture and thromboembolic models, functional outcome was poorer in diabetic animals. When post ischemia period was extended to 7 days, infarction expanded especially in the cortex reaching to levels observed in control animals suggesting that there is delayed neuronal death in diabetes. Diabetic animals persistently showed greater neurological deficit at day 7. This is consistent with previous clinical and experimental studies, which reported that greater infarct expansion contributes to unfavorable stroke outcomes in hyperglycemic patients (Baird et al., 2003).

Metformin is one of the most commonly used oral hypoglycemic agents to treat type 2 diabetes (Musi et al., 2002). Our previous study has shown that chronic metformin treatment inhibited diabetes-induced vascular remodeling and improved outcome after acute experimental stroke (Elgebaly et al., 2010). A recent study, however, showed that acute metformin treatment before brain ischemia worsens stroke injury and functional outcome in otherwise healthy animals (Li et al., 2010) due to activation of AMP-activated protein kinase (AMPK). In the current study, the acute metformin treatment in diabetic rats worsened the infarct size and HT, and caused significantly worse neurological deficiency as compared to untreated diabetic animals. Collectively, these studies suggest that acute metformin effects are independent of blood glucose lowering. Normalization of blood glucose with insulin showed a completely opposite effect suggesting that these results are specific for the metformin treatment. Insulin is the most commonly used agent to regulate blood glucose and has been shown to reduce ischemic brain damage (Siemkowicz et al., 1982; Warner et al., 1992; Wass and Lanier, 1996), while we cannot differentiate whether the protective effects observed in this study is due to glucose control or direct effects of insulin.

Conclusions and Limitations

There are several limitations that need to be recognized. First, this study used only male and young animals. Second, we used the GK model of diabetes which is developed from glucose intolerant Wistar rats (Goto et al., 1976). For this reason we have chosen to use age-matched Wistar rats as normoglycemic controls in these studies. Our finding that the neurovascular injury pattern in GK rats that present with normal glucose levels is similar to that observed in Wistar rats supports the use of Wistar rats as the control group. Glucose intolerance can be observed in GK rats as early as 4 weeks of age in these animals and onset of moderate hyperglycemia can be as early as 5–6 weeks of age (Goto et al., 1988). During weeks 6–12, plasma insulin levels are elevated and then decrease to levels lower than observed in Wistar rats (Goto et al., 1976; Harris et al., 2005). In contrast to the severely hyperglycemic streptozotocin rat model of diabetes, whose glucose levels are typically above 300 mg/dL (Kamada et al., 2007; Martini and Kent, 2007), GK rats are moderately hyperglycemic with plasma glucose levels similar to what has been observed in stroke patients (Bruno et al., 2008; Johnston et al., 2009; Kelly-Cobbs et al., 2012). Third, we did not use tPA in our experiments and studied spontaneous resolution of the clot. Nevertheless, we provide evidence that diabetes is associated with infarct expansion with an extended reperfusion period and there are differences in the neurovascular injury caused by suture versus thromboembolic stroke. The robust and rapid reperfusion achieved by mechanical removal of the suture causes greater bleeding in diabetic animals, an effect not seen in the thromboembolic model. We used a partially humanized clot that is supplemented by human fibrinogen and this model would be a powerful tool for future experiments focusing on the improvements in therapeutic strategies for diabetic stroke involving tPA therapy. While metformin had negative effects on the severity of neurovascular injury and functional outcome when used acutely, past studies and our findings with insulin and chronic use of metformin (Elgebaly et al., 2010; Prakash et al., 2013b) beyond the acute injury period emphasize the importance of glycemic control in the recovery from stroke.

4. Material and methods

4.1. Animal groups

The animals were housed at the Georgia Regents University animal care facility, which is approved by the American Association for Accreditation of Laboratory Animal Care. This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research and all protocols were approved by the institutional animal care and use committee. A total of 78 male 8–11 wk old control Wistar (Harlan Laboratories Inc., Indianapolis, IN) and diabetic Goto-Kakizaki (GK) rats (in house colony and Taconic, Hudson, NY) were randomly assigned to: group 1: 3 h ischemia and 21 h reperfusion (3–21); group 2: thromboembolic model (embolic) and the animals were sacrificed at 24 h time point after ischemia; group 3: 90 min ischemia and 23 h reperfusion (90–23); group 4: 3 h ischemia and 7 day reperfusion (3–7). To determine the impact of glycemic control, untreated diabetic GK rats were compared to GK rats with normal blood glucose level (GK NBG, n=10) that were screened from the in-house colony or to GK rats were treated with metformin (GK met, n=7, 300 mg/kg/day in drinking water) or insulin (GK ins, n=6, LinShin insulin pellets, 2 U/24 h) for 3 days (Elgebaly et al., 2011; Prakash et al., 2013a; Rizk et al., 2006). In this set of experiments, 3–21 h MCAO model as in group 1 was used.

4.2. Preparation of the embolus

The method of clot preparation was adapted from earlier reports and modified to increase the strength of uniformity of the fibrin rich core and stability of occlusion we recently

reported (Hoda et al., 2011; Zhang et al., 1997). 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 and retained in the tube for 6 h to clot at room temperature, and subsequently for 18 h at 4°C. The PE-50 tube containing clot was cut into 5 cm long pieces and attached to a 20 cm PE-10 tube interconnected by a syringe filled with saline. The clot was 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 modified PE-10 catheter for embolization.

4.3. Stroke surgery

Focal cerebral ischemia was induced by transient suture or embolic MCAO as previously described (Ergul et al., 2007; Zhang et al., 1997). Animals were anesthetized with 5% isoflurane, and anesthesia was maintained with 2.0% isoflurane in 70% N₂ and 30% O₂ using a face mask. In the suture model, a filament was inserted up to the origin of MCA through the stump of external carotid artery (ECA). The filament was left in and withdrawn from the vessel to reestablish blood flow at various time points as mentioned above. In the embolic model, a modified PE-10 catheter containing the clot was inserted through the ECA stump as in the suture model, 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 laser Doppler (PIM3, Perimed; North Royalton, OH) was used to confirm successful occlusion and ensure similar levels of blood flow reduction in all groups. The percent decrease of the cerebral blood flow compare to the baseline before occlusion was reported.

4.4. Infarct size, edema ratio, and hemorrhagic transformation analysis

After sliced and labeled from A to G (+6.7 mm to -8.8 mm of Bregma), the infarct size of the brain was measured after 2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described (Ergul et al., 2007). Edema was calculated as a percent (%) increase in the ischemic hemisphere vs. the contralateral hemisphere, and the edema ratio was reported after normalizing for infarct size. After staining, the hemispheres were separated and deep frozen for tissue hemoglobin quantification with QuantiChrom kit (BioAssay Systems, Hayward, CA) (Qin et al., 2007) and reported as excess hemoglobin (Hb, mg/g protein) in the ischemic hemisphere (ischemic hemisphere minus contralateral hemisphere). A blinded investigator scored macroscopic HT in brain slices B to E using a four-point rubric (Kelly-Cobbs et al., 2013) (0 – Normal ischemic damage or 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.

4.5. Behavioral measurements

Neurobehavioral tests were assessed, recorded and scored in a blinded fashion as described before (Prakash et al., 2013b). Animals were handled for 5–7 days prior to behavior testing in rooms where behavior testing was to be carried out. Tests included Bederson's score, beam walk and grip strength tests before and after stroke at 24 h and 7 days prior to sacrifice. Bederson's score for each rat was obtained by using 3 parameters which include (a) observation of spontaneous ipsilateral circling, graded as 2 (no circling), 1 (partial circling), 0 (continuous circling), (b) contralateral hindlimb retraction, (c) forelimb retraction which measures the ability of the animal to replace the limb after it is displaced laterally by 2 to 3 cm, graded 1 (immediate replacement) and 0 (replacement after minutes or no replacement). Maximum score of 5 was allotted to a normal rat. Beam walking ability graded based on 7-point scale method as described by Feeney et al. (Feeney et al., 1982; Kelly-Cobbs et al., 2013). Composite neurological scores composed of sum of the Bederson's score and the scores obtained from the beam walking test. Forelimb grip strength

was measured with a standard grip strength meter (Columbus Instrument, Columbus, OH) (Terry et al., 2007).

4.6. Statistical Analysis

Data were evaluated for normality and appropriate transformations were used when necessary. Data are expressed as mean $\pm$ SEM or shown in scattered plots with median where appropriate. Wilcoxon rank-sum (Mann-Whitney) test was used to analyze the differences between control and diabetic animals in each stroke model. A one-way ANOVA was used to study the impact of glycemic control on stroke outcomes. Statistical significance was determined at $\alpha=0.05$. SAS® version 9.3 was used for all analyses (SAS Institute, Inc., Cary, NC).

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Abbreviations

AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
BBB	blood-brain barrier
ECA	external carotid artery
GK	Goto-Kakizaki
HT	hemorrhagic transformation
I/R	ischemia/reperfusion
MCAO	middle cerebral artery occlusion
met	metformin
NBG	normal blood glucose
PE	polyethylene
SEM	standard error of the mean
STAIR	Stroke Therapy Academia Industry Roundtable committee
tPA	tissue plasminogen activator
TTC	2,3,5-triphenyltetrazolium chloride

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Fig 1.

The effect of diabetes on infarct size in different stroke models. 3–21: 3 h ischemia and 21 h reperfusion; embolic: thromboembolic model; 90–23: 90 min ischemia and 23 h reperfusion; 3–7: 3 h ischemia and 7 day reperfusion. A. Representative images of infarct in each model. White areas indicate infarct. B. Scatter plot demonstration all data points with median in each experimental stroke model. Values are compared between Wistar and GK groups by Wilcoxon rank-sum test. C. Changes in cerebral blood flow after occlusion was similar across experimental stroke models.

Fig. 2.

The effect of diabetes on neurovascular injury in different stroke models. 3–21: 3 h ischemia and 21 h reperfusion; embolic: thromboembolic model; 90–23: 90 min ischemia and 23 h reperfusion. A Representative images of unstained brain slices in Wistar and GK rats. HT can be seen as spotted or diffused petechiae. B. Edema ratio was calculated as the ratio of edema and infarct size. HT index (C) and tissue Hb (D) was measured as indices of macroscopic bleeding and bleeding severity, respectively. Data as shown as scatter plot with median to clearly demonstrate the variation within each group.

Fig. 3.

The effect of diabetes on neurological deficit in different stroke models. 3–21: 3 h ischemia and 21 h reperfusion; embolic: thromboembolic model; 90–23: 90 min ischemia and 23 h reperfusion; 3–7: 3 h ischemia and 7 day reperfusion. Grip strength (A) and composite score (B) was lower in diabetic animals regardless of the method of stroke. * $p < 0.05$ vs. Wistar.

Fig. 4.

The effect of acute blood glucose control on neurovascular injury. GK met: metformin treated GK rats; GK ins: insulin treated GK rats; GK NBG: GK rats with normal blood glucose level. A. Representative images of stained brain slices showing the infarct. B. Metformin treatment increased the infarct size. * $p < 0.05$ vs. GK. C. Edema ratio was lower in GK rats that present with normal blood glucose levels. ** $p < 0.05$ vs. GK.

Fig. 5.

The effect of acute blood glucose control on hemorrhagic transformation. GK met: metformin treated GK rats; GK ins: insulin treated GK rats; GK NBG: GK rats with normal blood glucose level. A. Representative images of unstained sections in each group. B. HT index was lower in insulin treated or normal blood glucose GK rats. * $p < 0.05$ vs. GK. C. Excess Hb was increased in metformin-treated animals. $p < 0.05$ vs. other groups.

Fig. 6.

Neurological deficit in GK rats with acute blood glucose control treatment. GK met: metformin treated GK rats; GK ins: insulin treated GK rats; GK NBG: GK rats with normal blood glucose level. A. Grip strength was lower in metformin-treated animals but better in insulin treatment or normal blood glucose GK groups. * $p < 0.05$ vs. all other groups, ** $p < 0.05$ vs. GK rats. B. GK rats with normal blood glucose exhibited the best composite score. * $p < 0.05$ vs. all other groups.

Table 1

Physiological parameters in each ischemia/reperfusion model. 3–21: 3 h ischemia and 21 h reperfusion; embolic: thromboembolic model; 90-23: 90 min ischemia and 23 h reperfusion; 3–7: 3 h ischemia and 7 day reperfusion.

Model	Strain	Body weight (g)	Blood glucose (mg/dL)
3–21	Wistar	287±14	92±2
	GK	266±14	159±12 [*]
Embolic	Wistar	296±12	88±4
	GK	267±6	156±11 [*]
1.5–22.5	Wistar	308±6	74±3
	GK	283±12	244±11 ^{**}
3–7	Wistar	290±6	90±6
	GK	273±13	135±14 [*]

^{*} p<0.05 vs. Wistar,

^{**} p<0.05 vs. all other groups.

Table 2

Physiological parameters in glucose control group. GK met: metformin treated GK rats; GK ins: insulin treated GK rats; GK NBG: GK rats with normal blood glucose level.

Treatment	Body weight (g)	Blood glucose (mg/dL)
GK	266±14	159±12 [*]
GK met	251±8	104±3
GK ins	271±1	76±7
GK NBG	283±9	103±5

Diabetes worsens functional outcome regardless of the method used to induce stroke.

Diabetes accelerates development of hemorrhagic transformation.

There is greater infarct expansion in diabetes.

Acute metformin treatment worsens vascular injury after stroke in diabetes.

^{*}
p<0.05 vs. all other groups.