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## Neurovascular injury in acute hyperglycemia and diabetes: A comparative analysis in experimental stroke

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### Abstract

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

**Purpose**—To determine the effect of acute HG on neurovascular outcomes of stroke under control and diabetic conditions.

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

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

**Conclusions**—The finding that functional outcome is poorer in both acute HG and diabetes without a significant increase in infarct size suggests that amplified vascular damage contributes to neurological deficit in hyperglycemia. These results highlight the importance of vascular protection to improve neurological outcome in acute ischemic stroke.

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## Keywords

MMP-9 Metalloproteinase; Minocycline; Cerebral Hemorrhage; Ischemia-Reperfusion Injury; Type 2 Diabetes Mellitus; Hyperglycemia

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## Introduction

Ischemic stroke is a leading cause of death and disability in the United States and diabetes is the most rapidly increasing risk factor for stroke (1). Stroke risk in patients with diabetes is 2-6 folds higher than age-matched controls (1). The short and long term functional outcomes are worse and mortality is greater in stroke patients with diabetes as compared to the non-diabetic population (2). In addition to increased risk for stroke with diabetes, acute hyperglycemia (HG), which can develop as a stress response exacerbates stroke (3-5). However, it is not clear whether there is a difference between the effect of HG versus diabetes on clinical stroke outcomes (6). This is clinically important as the current stroke guidelines emphasize the need for randomized controlled trials to determine best practice for managing hyperglycemia (7). The GIST-UK randomized clinical trial (8) enrolled predominantly patients without diabetes (83%). Although insulin achieved target blood glucose levels, there was no difference in stroke outcomes. Results from THIS (9) and GRASP (10) pilot trials, both of which enrolled mainly diabetic patients (>50%), have suggested a favorable outcome in patients rapidly treated for hyperglycemia. While needing confirmation by larger trials, these results highlight the importance of targeting the right patient populations and the right blood glucose range to be achieved clinically.

Previous preclinical studies on diabetic and hyperglycemic ischemic brain injury do not provide sufficient information as most studies employed HG induced by either glucose injection or streptozotocin (STZ) injection 2-3 days prior to stroke with very high blood glucose levels (11-13). However, recent clinical evidence suggests that blood glucose levels in stroke patients that present with hyperglycemia at admission range between (140-200 mg/dl) (6, 9, 14). Understanding the mechanisms behind diabetes versus HG-dependent stroke pathology in animal models that closely represent the clinical condition is paramount in improving current preventive and therapeutic interventions.

Matrix metalloproteases (MMP) play an important role in vascular remodeling as well as stroke pathophysiology (15-17). We previously reported that augmented cerebrovascular MMP-2 and MMP-9 activity in the Goto-Kakizaki (GK) model of type-2 diabetes is associated with enhanced remodeling (18) and increased hemorrhagic transformation (HT) following ischemic stroke (19). Clinically, plasma MMP-9 levels are predictive of HT following acute ischemic stroke (16, 20). However, regulation of cerebrovascular and plasma MMP-9 levels in hyperglycemic and diabetic models and the subsequent effects on stroke pathology remain unknown.

Building upon these past findings, this study was designed to address the questions: 1. How do moderate acute hyperglycemia and diabetes affect neurovascular damage and stroke outcome?, 2. Does a further acute elevation in blood glucose at the time of ischemia exacerbate neuronal and vascular injury in diabetes?, and 3. How do HG and diabetes influence vascular and plasma MMP-9 following ischemic stroke?

## Materials and methods

### Animal models

The institutional animal care and use committees (IACUC) of the Medical College of Georgia and the Charlie Norwood Veterans Affairs approved all protocols. Male Wistar and GK rats were from Harlan (Indianapolis, IN) and Taconic (Hudson, NY), respectively, and weighed (260-310 g). Study groups included 1) Control, 2) Control + acute HG, 3) Diabetes, and 4) Diabetes + acute HG. Acute HG was achieved by a 3 ml IP glucose injection (300 mmol) 20-30 minutes before MCAO and was maintained during ischemia by another injection 1.5 h after MCAO. Two additional groups were: 1) Diabetic rats treated with metformin (300 mg/kg/day) starting at the onset of diabetes till MCAO and 2) Diabetic rats treated with metformin and given acute HG during MCAO. The purpose of the last 2 groups was to determine 1) whether glycemic control prevents/reduces injury if a stroke is superimposed on diabetes, and 2) whether an acute elevation in blood glucose cause the same damage if there was prior glycemic control in diabetes. Metformin was given in drinking water artificially sweetened by non-caloric sweetener for the 4 weeks before the study. Blood glucose was measured from tail vein using a glucometer (Freestyle, Alameda, CA) and reported at baseline, MCAO surgery and sacrifice. MCAO blood glucose levels were given as the average of readings at induction of anesthesia and 1h MCAO.

### Experimental temporary focal cerebral ischemia

3 h MCAO was performed under 3% isoflurane anesthesia followed by 21 h reperfusion using a modified Longa method as we reported before (19). Scanning laser Doppler (Pim-3, Perimed, ST) was used to confirm a similar degree of drop in flow between groups. Core body temperature was monitored rectally and maintained using a heating pad under the animal and under the cage till the end of 24 h. Animals were singly housed before and after MCAO with free access to food and water. In a subset of animals, arterial blood gases were measured before and after MCAO as well as post-reperfusion via femoral artery catheterization.

### Evaluation of neurovascular injury

Before sacrifice animals underwent intracardiac perfusion of ice-cold saline to flush blood from the vessels. Brains were extracted and MCAs were isolated from ischemic and nonischemic hemispheres. Visual inspection of hemorrhage was done, documented per slice if present and reported in a binary fashion as yes or no to indicate the frequency of macroscopic bleeding. 2, 3, 5-triphenyltetrazolium chloride (TTC) was used to outline the infarct area. Images were analyzed in a blinded fashion using Image-J. Edema is reported as the percent increase in ischemic hemisphere size to the contralateral hemisphere. Infarct volume is reported as the % infarcted area of ipsilateral hemisphere corrected for edema. Indirect infarct size was also calculated as contralateral area minus the area of noninfarcted tissue in the ischemic hemisphere. Following staining, hemispheres were separated and stored at  $-80^{\circ}\text{C}$  for later hemoglobin (Hb) ELISA. HT was quantified by measuring excess Hb in the stroked hemisphere compared to the contralateral side as previously published (21).

### Evaluation of MMP activity

For vascular MMP-9 activity, MCAs isolated from ischemic and nonischemic hemispheres were homogenized as reported previously (18) and 30  $\mu\text{g}$  homogenate or 20  $\mu\text{l}$  (1:20 diluted) plasma were assayed by gelatin zymography as we reported previously (19). Briefly, samples were loaded directly on SDS-PAGE gel containing 0.1% gelatin and separated under non-reducing conditions. Following electrophoresis, the gel was washed twice in

2.5% “Triton X100” for 20 minutes each, rinsed with ddH<sub>2</sub>O and incubated for 20 h in a substrate buffer “50 mmol Tris-HCl, 5 mmol CaCl<sub>2</sub> + 0.02% NaN<sub>3</sub>, pH= 7.5” at 37°C. Recombinant MMP-9 active standard was run as positive control (Calbiochem, catalogue# PF024). Following incubation, the gel was stained using “Coomassie blue” for 3 h then destained. The zymogram was digitized and band intensity was quantified by image analysis “GelPro analyzer, Media Cybernetics, MD” and expressed as the % of the recombinant standard.

### Evaluation of neurological outcome

Ipsilateral circling, paw grasp and beam walk tests assessed function at baseline, before MCAO and at 24 h. Scores were graded 0-3 (Normal-maximum deficit). Ipsilateral circling (No/ few/ several and continuous circling), paw grasp (Normal grasp/ stroke side can touch/ stroke side hard to touch and unable to touch), beam walk (Animal readily traverses/ walks slowly and shaking/ can stay or eventually falls and unable to stay for 10 seconds). A total score of 9 indicated the worst outcome. Bederson’s score was recorded at baseline and after occlusion to verify proper occlusion (22).

### Statistics

The distributions for the measures of stroke severity (Infarct size, percent edema, and bleeding) as well as the measures of behavior were found to be skewed. A rank transformation was used prior to the analysis of these measures. The analysis for the effect of hyperglycemia on Wistar and GK rats was performed using a 2 disease (Wistar vs GK) X 2 treatment (Vehicle vs hyperglycemia) ANOVA. An interaction between disease and treatment would indicate a differential effect of inducing hyperglycemia that is dependent on disease status. The analysis for the effects of hyperglycemia and metformin was performed using a 2 hyperglycemia (No vs yes) X 2 metformin (No vs yes) factorial ANOVA. An interaction between hyperglycemia and metformin would indicate a differential effect of metformin treatment dependent on hyperglycemic status. A Tukey’s test was used to adjust for multiple comparisons when determining mean differences for significant interactions.

## Results

### Physiological parameters

Blood glucose levels over the course of MCAO/reperfusion are depicted in Fig. 1a. During ischemia, we achieved 1) A blood glucose range in control rats similar to the average blood glucose values in diabetic GK rats (140-200 mg/dl), and 2) An acute increase in blood glucose in diabetic rats. Baseline blood glucose was significantly higher in diabetic than in control rats. In all groups, blood glucose increased during MCAO and diabetic rats showed a greater elevation in blood glucose compared to control + HG group (Fig. 1a). The purpose of the second glucose IP injection midway during occlusion was to maintain HG during MCAO. At sacrifice, blood glucose dropped to similar level in all groups. Metformin corrected hyperglycemia in diabetic rats. Glucose injection achieved a similar degree of increase in blood glucose in both vehicle and metformin treated diabetic rats at baseline (Fig. 1b). At sacrifice, blood glucose dropped to control levels in all groups (Fig. 1a).

Arterial blood gases measurements were similar between control and diabetic rats before and after MCAO as well as post-reperfusion (Table 1).

### Neurovascular damage

Infarct size was significantly lower in diabetic rats than in controls as we previously reported (Fig. 2a and b) (19). Indirect infarct size measurements showed similar results (Not shown). There was a disease and treatment interaction such that HG increased infarct size in

diabetic (30 vs 10%) but not in control (30%) (Fig. 2b). Metformin pretreatment did not affect infarct size in diabetic rats with or without additional acute HG (Fig. 2b). The percent reduction in blood flow from baseline after MCAO was similar in all groups indicating consistent occlusion (Fig. 2c).

Edema and hemorrhagic transformation (HT) were analyzed as indices of vascular damage. HT was assessed qualitatively by observing macroscopic hemorrhagic transformation and quantitatively by Hb ELISA (ng/mg protein) (Fig. 3b and c). Diabetic rats had a higher rate of HT than the controls (87% vs 15%,  $p < 0.001$ ). Excess Hb and edema were higher in diabetes indicating vascular damage and there was a disease and treatment interaction such that HG increased both parameters in control but not diabetes (Fig. 3c and d). Metformin reduced brain edema in diabetic rats (Fig. 3d). While it did not affect the rate of HT, bleeding severity was reduced (Fig. 3c).

### MMP activity

MMP-9 is associated with disruption of vascular integrity in ischemic stroke. Both MCA and plasma MMP-9 activity were measured (Fig. 4). MCA MMP-9 activity was greater in the nonischemic hemisphere of diabetic rats than in control (Fig. 4a). Ischemia enhanced MCA MMP-9 activity in both control and diabetic rats. Metformin pretreatment did not affect MMP-9 activity on the nonischemic side but prevented the increase on the ischemic hemisphere. HG did not change MCA MMP-9 activity. Plasma MMP-9 activity at baseline was higher in diabetes (Fig. 4b). MCAO caused a dramatic increase versus baseline in all groups.

### Neurological outcome

Short term (24 h) outcomes were assessed. Neurological deficits were more pronounced in diabetes rats and HG worsened functional outcomes in both control and diabetic rats (Fig. 5). Metformin improved the deficit in diabetic animals.

### Discussion

This study provides several important lines of evidence on the effects of acute hyperglycemia and diabetes (chronic hyperglycemia) on neurovascular and functional outcomes of stroke: 1) Moderate acute hyperglycemia amplifies vascular damage (edema and HT) without increasing infarct size following stroke in otherwise healthy rats. 2) Moderate diabetes is associated with smaller infarcts but greater edema and HT. Overlaying acute hyperglycemia in diabetes exacerbates infarction but does not amplify the HT or edema that is already greater in diabetic rats compared to control rats. 3) Upregulation of MMP-9 activity is a mechanism associated with increased vascular damage in both acute hyperglycemia and diabetes. 4) Glycemic control provides vascular protection in diabetes. The interesting finding that functional outcomes are worse in acute hyperglycemic and diabetic rats despite relatively smaller infarcts strongly suggests that vascular injury contributes to the neurological deficits.

Diabetes and HG are predictors of poor ischemic stroke outcomes. Yet, limited knowledge exists about the different pathophysiology, outcomes and the mechanisms of injury in each case (6, 23). Clinical findings link admission HG, independent of diabetes, to poor outcomes and to increased odds of HT (24, 25). In experimental models, larger infarct size and higher mortality rates were reported after hyperglycemic ischemic stroke. Most of these studies employed acutely-induced severe hyperglycemia (26, 27). Furthermore, diabetes was induced for only a short period of time prior to MCAO rather than being a fully developed disease state (11). Our study expands our current knowledge as GK rats present with mild-

moderate hyperglycemia (~ 180 mg/dl) which more closely resembles the clinical situation where admission hyperglycemia is defined as glycemia >140 mg/dl (6, 9, 14). This is in comparison to the STZ model with severe glycemia (~500 mg/dl). In addition, GK rats have been moderately diabetic for a relatively longer period of time, about 5-6 weeks before MCAO.

The infarct size in our diabetic rats was smaller than control as we previously reported (19, 28, 29). This may be due to diabetes-mediated stimulation of survival mechanisms as a compensatory response early in the disease process (30). We had hypothesized that if diabetes activates survival pathways in GK rats, then glucose control would prevent this effect and GKs would develop infarct sizes similar to control. However, there was no significant change in infarct size due to metformin pretreatment. This would suggest that the smaller infarcts in GK are either not due to diabetes-mediated changes in cell survival or that this is a metformin-specific protective effect. Recent studies reported that metformin may also be neuroprotective (31, 32). Future studies are needed to address the preconditioning possibility in GK rats in more depth. At the levels of acute hyperglycemia achieved in our study, ischemic damage in control HG rats was not different from control normoglycemic animals. Yet, when acute HG was superimposed on diabetic rats, ischemic damage increased. This finding may be due to the fact that while baseline blood glucose was similar between control + HG and diabetes groups at the beginning of stroke surgery, diabetic animals showed a greater spike during MCAO indicating a greater stress response. While the reasons are not completely delineated, acute HG in human stroke may be due to stress response by activation of the sympathetic system or the hypothalamic-pituitary-adrenal axis. In our model acute HG was induced by glucose injection but the role(s) of these systems cannot be excluded in our findings.

Neurovascular unit integrity is compromised following prolonged ischemia causing edema and HT due to disruption of the tight junction proteins and vessel breakdown. Admission blood glucose is a predictor of HT in patients given t-PA (3-5). Thus, controlling HG in ischemic stroke is likely to be important in maximizing the therapeutic potential of t-PA. In our study, 87% of diabetic rats exhibited intracerebral bleeding while this was 100% in HG animals versus 15% in control. Upon quantification, severity of HT was higher in diabetic rats compared to control. Edema was higher in diabetic and HG rats compared to control, suggesting that cerebrovasculature is susceptible to damage by hyperglycemia regardless of diabetes. It also points out that the threshold for vascular damage is relatively low and mild-moderate levels of HG are sufficient to induce it.

Upregulation of MMPs may be a common link between diabetes and ischemic stroke pathologies. Increased MMP-9 disrupts basal lamina and neurovascular unit structure promoting edema and HT in ischemic stroke (16, 17, 33-35). MMP-9 knockout mice were shown to be protected against ischemic stroke. It is postulated that the HT complicating tissue plasminogen activator (t-PA) therapy is mediated through upregulation of MMP-9 (36). We investigated the parallel patterns of MMP-9 activity in both cerebral macrovessels and in plasma (tissue versus circulation) in diabetes and HG. Baseline plasma MMP-9 activity was upregulated in diabetes compared to control. At 24 h, MMP-9 plasma activity in all groups increased significantly up to similar level due to the ischemic injury. A similar pattern was observed in macrovessels where type-2 diabetes upregulated MCA MMP-9 activity compared to control in the non-stroke side and ischemic injury caused a further elevation of MMP-9 activity in both control and diabetes macrovessels on the stroke side. The fact that bleeding frequency was significantly higher in both diabetes and HG rats than in controls, yet the 24 h MMP-9 activity was not different between groups, indicates that earlier time points of plasma MMP-9 activity need to be investigated for proper correlation



between plasma MMP-9 activity and HT for optimal utilization of MMP-9 as an ischemic stroke biomarker (37).

Our study assessed neurological function at 24 h while the damage is still evolving. The finding that the deficit is greater in the diabetic versus control groups and that HG exacerbated the neurological deficit in both control and diabetic rats suggests that both acute hyperglycemia and diabetes are critical for stroke outcome. An interesting finding is that while glycemic control with metformin did not affect infarct size (Fig. 2b), it improved neurological outcome in diabetes (Fig. 5). Pretreatment with metformin also reduced the deficit mediated by HG in diabetic rats. A novel aspect of the current study is that the functional outcome was worse in both acute and chronic hyperglycemia despite relatively smaller infarct sizes suggesting that vascular injury and potentially subsequent vascular dysfunction in these groups may be as important as the neuronal injury in determining neurological deficit. However, it is recognized that short term 24 h measurement is a limitation of the study and more sensitive methods and longer term outcomes are needed for confirmation.

## Conclusion

Therapeutic options for ischemic stroke patients are limited. Given that 40-50% of acute ischemic stroke patients present with admission HG, understanding the mechanisms of neurovascular injury in acute HG and diabetes is very important. While the complexity of human stroke cannot be duplicated in experimental models, these preclinical studies are critical to guide the clinical studies to develop better prevention and intervention strategies in the management of acute ischemic stroke.

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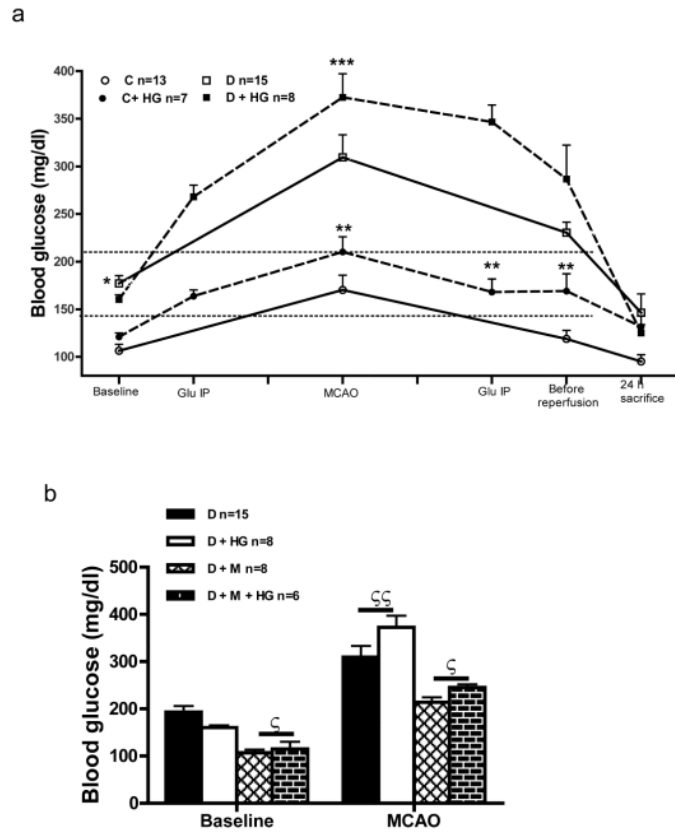
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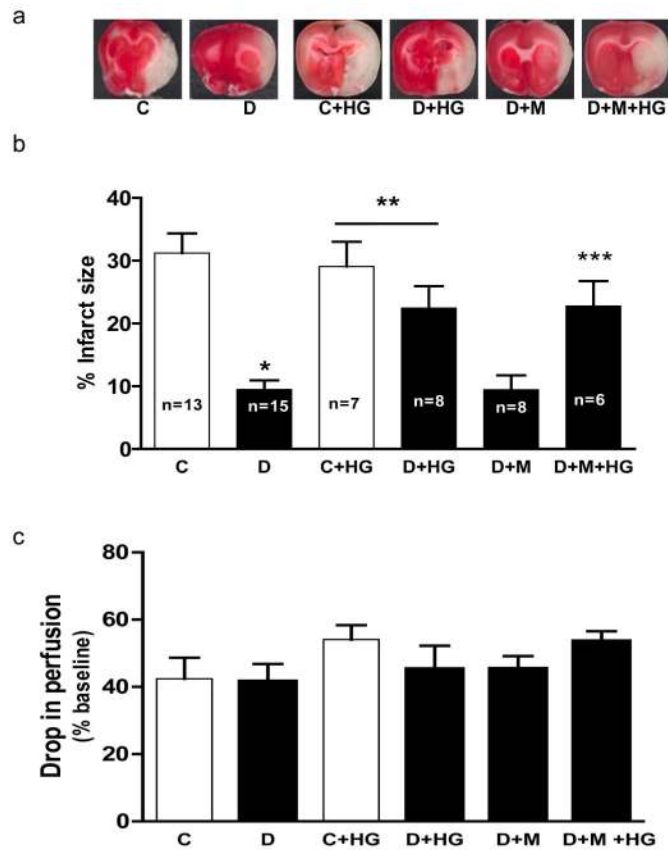
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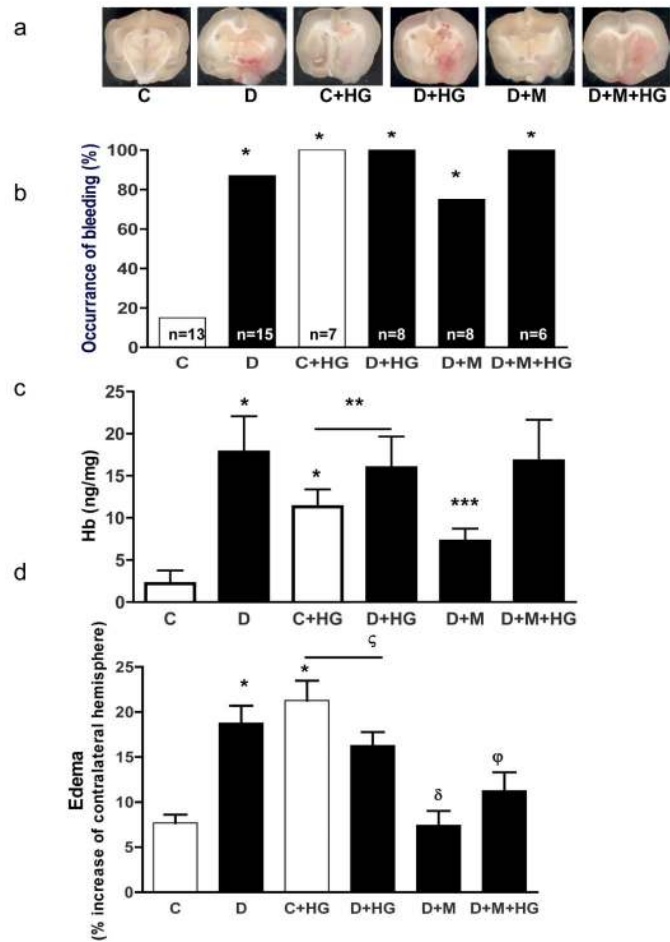
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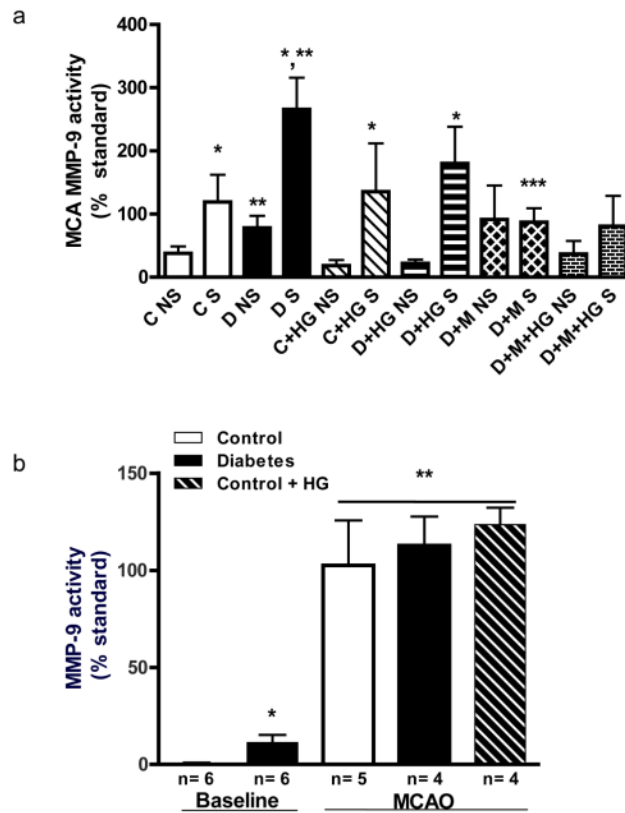
**Fig. 1.** (A) Blood glucose at baseline and during MCAO in Control (C), C+ High Glucose (HG), Diabetes (D), and D+ HG groups. (B) Effect of glycemic control with metformin (M) on blood glucose at baseline and during MCAO. \* $p < 0.05$  vs C or C +HG, \*\* $p < 0.05$  vs C, D or D + HG, \*\*\* $p < 0.05$  vs D,  $\zeta p < 0.05$  vs D or D + HG,  $\zeta\zeta p < 0.05$  vs baseline. Glu IP= glucose intraperitoneal injection (Dotted horizontal line indicates the range of achieved HG).



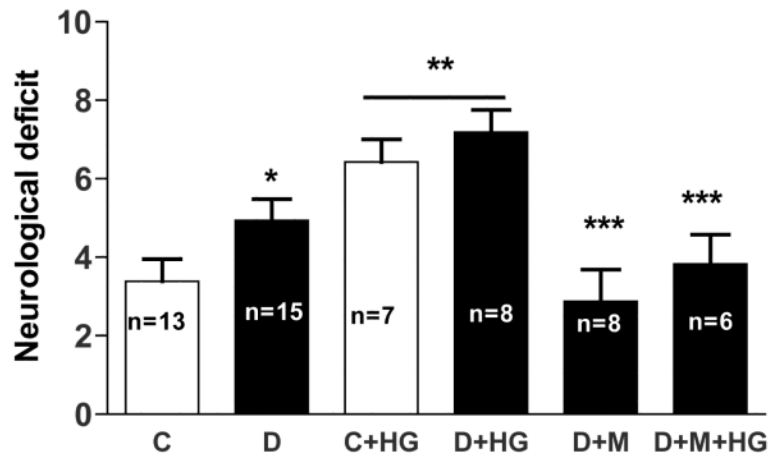
**Fig. 2.** HG increases infarct size when superimposed on diabetes. Representative images of ischemic damage after TTC stain and quantitative analysis of infarct size are shown in panels **A** and **B**, respectively. **(C)** Drop in flow following MCAO was similar among groups. \* $p < 0.05$  vs C, \*\* $p = 0.0062$  disease by treatment interaction compared to C and D and \*\*\* $p = 0.0035$  vs D or D + M.



**Fig. 3.** HG and diabetes augment vascular damage. **(A)** Representative images of hemorrhagic transformation (HT). **(B)** Frequency of macroscopic HT is significantly higher in diabetes and HG versus control. Severity of bleeding **(C)** and edema **(D)** are greater in diabetes and HG. **(D)**. \* $p < 0.05$  vs C, \*\* $p = 0.016$  disease by treatment interaction compared to C and D,  $p < 0.001$  vs D, \*\*\* $p = 0.0089$  vs D,  $\zeta p < 0.0001$  disease by treatment interaction compared to C and D,  $\delta p < 0.0001$  vs D and  $\phi p < 0.01$  vs D or D + HG.



**Fig. 4.** Local MCA and circulating MMP-9 activity under basal and ischemic conditions. **(A)** MCA MMP-9 lytic of non-stroke (NS) and stroke (S) hemispheres by gelatin zymography. Baseline (NS) activity was greater in diabetes than in control and further increased with ischemia. **(B)** Baseline plasma MMP-9 activity was higher in diabetes and at 24 h after MCAO both diabetes and HG caused a significant increase. \* $p < 0.05$  vs NS, \*\* $p < 0.001$  vs C S and \*\*\* $p < 0.001$  vs D S.



**Fig. 5.** Effect of HG and diabetes on 24 h neurobehavioral outcomes. A greater composite score indicates greater neurological deficit and poor outcome. \* $p < 0.05$  vs C, \*\* $p < 0.0001$  compared to C and D and \*\*\* $p < 0.001$  vs D or D + HG



Table 1

Arterial blood gases before and after MCAO.

	Control <sup>a</sup>			Diabetes <sup>a</sup>		
	before	after	postRe <sup>b</sup>	before	after	postRe <sup>b</sup>
pH	7.45±0.01	7.45±0.02	7.40±0.02	7.42±0.02	7.39±0.01	7.39±0.03
pCO <sub>2</sub>	41.37±0.83	40.78±2.10	49.12±2.54	48.68±2.46	51.61±2.60	47.91±2.88
pO <sub>2</sub>	163.83±1.5	168.50±3.5	120.20±11.1	150.18±4.1	161.00±4.5	133.43±9.9
n	6	4	5	11	8	7

<sup>a</sup>Body temperature 37 ± 0.5 C<sup>0</sup>.<sup>b</sup> postRe = post reperfusion.