Original Contribution

Persistent Cerebrovascular Damage After Stroke in Type Two Diabetic Rats Measured by Magnetic Resonance Imaging

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Background and Purpose—Diabetes mellitus is a disease with vascular components. Consequently, the blood–brain barrier disruption after stroke may differ between diabetic and nondiabetic animals. However, few studies have documented the longitudinal blood–brain barrier disruption after stroke in diabetic animals. In this study, using MRI, we noninvasively evaluated the blood–brain barrier disruption after middle cerebral artery occlusion in diabetic and nondiabetic rats.

Methods—Type 2 diabetes mellitus (T2DM) was induced in adult male Wistar rats by administration of a high-fat diet in combination with a single intraperitoneal injection (35 mg/kg) of streptozotocin. T2DM rats (n=9) and nondiabetic wild-type (WT) rats (n=9) were subjected to middle cerebral artery occlusion for 2 hours using the filament model. MRI was performed 1 day and then weekly for 5 weeks after middle cerebral artery occlusion for all rats.

Results—The ischemic lesion volumes after stroke as measured using T2 maps were not significantly different between the T2DM and WT rats. Compared with the WT rats, the volumes of blood–brain barrier disruption evaluated using contrast-enhanced T1-weighted imaging with gadolinium-diethylenetriamine penta-acetic acid and the cerebral hemorrhagic volumes measured with susceptibility-weighted imaging were significantly (P<0.05) larger in the T2DM rats from 1 to 5 weeks after stroke; values of diffusion fractional anisotropy were significantly lower in T2DM rats (P<0.03) than in WT rats after stroke. These MRI measurements were consistent with histological data.

Conclusions—Using MRI, T1-weighted imaging did not detect significant differences of the ischemic lesion volumes between T2DM and WT rats. In contrast to the WT rats, however, contrast-enhanced T1-weighted imaging and susceptibility-weighted imaging identified much more severe ischemic vascular damage, whereas fractional anisotropy demonstrated lower axonal density in the T2DM rats after stroke. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.007538.)

Key Words: blood–brain barrier ■ diabetes mellitus ■ hemorrhage ■ magnetic resonance imaging ■ rats ■ stroke

Diabetes mellitus is a chronic vascular disease. Hyperglycemia induces a variety of biochemical changes within endothelial cells, including those in the cerebral vasculature. Diabetes mellitus instigates a cascade of events leading to vascular endothelial cell dysfunction and increased vascular permeability in various vascular beds in humans and animal models. Many pathways are involved in the diabetes mellitus–related changes in the blood–brain barrier (BBB). In the clinic, the vast majority (90%–95%) of diabetic patients have type 2 diabetes mellitus (T2DM), which affects 24 million Americans.

Diabetes mellitus increases risk of ischemic stroke more than hemorrhagic stroke. Clinical and experimental results have demonstrated that diabetes mellitus also increases stroke recurrence and long-term mortality from stroke and worsens the overall neurological outcomes after stroke. Abnormalities in glucose metabolism and vascular hemodynamics may play important roles in the pathogenic progress of stroke in diabetic patients.

Experimental studies have reported inconsistent ischemic lesion volumes in diabetic rodents compared with nondiabetic rodents, which may depend on the type and duration of diabetes mellitus, ischemia model, or the murine strain. Here, we used MRI to longitudinally measure the ischemic lesion volumes using a filament model of stroke in adult rats with or without T2DM induced by streptozotocin combined with a high-fat diet. \( ^{10,11} \) BBB damage and exacerbated secondary hemorrhagic transformation (HT) are consistent consequences of ischemic stroke in diabetic murine animals. However, prior preclinical studies only focused on the measurement of BBB disruption and cerebral vascular permeability rate at an early stage after stroke in diabetic animals using histological methods, which do not allow dynamic evaluation and application to patients. In the present study, using MRI, the temporal...
characteristics of BBB disruption were monitored weekly ≤5 weeks after stroke in the T2DM and nondiabetic wild-type (WT) rats. These results may provide new information on dynamic and chronic cerebrovascular damages after stroke in T2DM rats.

Materials and Methods

All experimental procedures were conducted and performed in accordance with guidelines for animal research under a protocol approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

Animal Model and Experimental Protocol

T2DM was induced in adult (175 g; 2–3 mol/L) male Wistar rats (Charles River, Wilmington, MA) by feeding them a high-fat diet (40% of calories as fat) for 2 weeks, and then injecting a single intraperitoneal dose, 35 mg/kg, of streptozotocin (Zanosar, Sigma Chemical Co, St. Louis, MO), a naturally occurring chemical that is particularly toxic to the insulin-producing beta cells of the pancreas in mammals, the high-fat diet was continued for another 2 weeks.

Blood glucose level was measured using test strips for glucose (Polymer Technology System, Indianapolis, IN) for confirmation of hyperglycemia (449.2±42.6 and 414.2±134.6 mg/dL at 1 day before and after middle cerebral artery occlusion (MCAo)). Right middle cerebral artery occlusion was then induced for 2 hours using the filament model, as previously described. Briefly, a 4.0 monofilament nylon suture, its tip rounded by heating, was introduced into the internal carotid artery lumen through the stump of the external carotid artery and gently advanced into the internal carotid artery 19 to 21 mm past the common carotid artery bifurcation to block the origin of the middle cerebral artery. Reperfusion was initiated through removal of the thread and tying off the distal external carotid artery. Wistar rats, fed normal Chow (12% of calories as fat) without streptozotocin injection, received suture-induced ischemia–reperfusion injury and were used as the WT control rats (blood glucose level, 98.3±2.1 and 90.8±5.6 mg/dL at 1 day before and after MCAo). The control and T2DM animal groups were age matched.

MRI was performed before the surgery for MCAo, as an internal control. Then, MRI was performed at 1 day and then weekly for 5 weeks after ischemia–reperfusion for all rats. Three T2DM rats and 1 WT rat died after MCAo and were excluded from the study. After completing MRI scans, all animals (n=9 for T2DM and n=9 for WT rats) were euthanized 5 weeks after stroke.

MRI Measurements

MRI measurements were performed with ClinScan 7T system, which combines Bruker-Biospin hardware (Bruker-Biospin, Ettlingen, Germany) with Siemens software (Siemens, Erlanger, Germany). A birdcage type coil was used as the transmitter and a quadrature half-volume coil as the receiver. Pulse sequences included T$_1$-weighted imaging, susceptibility-weighted imaging (SWI), diffusion-weighted imaging with multiple directions, and contrast-enhanced T$_2$-weighted imaging (CE-T1WI) with gadolinium-diethylenetriamine penta-ace­tic acid (Gd-DTPA; Magnevist, Berlex Inc, Montville, NJ), as the imaging contrast agent.

A fast gradient echo imaging sequence was used for reproducible positioning of the animal in the magnet at each MRI session. During MRI measurements, anesthesia was maintained using medical air (1.0 L/min) with isoflurane (1.0%–1.5%). Stereotactic ear bars were used to minimize movement, and rectal temperature was maintained at 37±1.0°C using a feedback-controlled water bath (YSI Inc, Yellow Springs, OH).

T$_2$-weighted imaging was acquired using a multislice (13 slices) and multiecho (6 echoes) sequence, with time of echo as 15 ms, and equal to 90 ms. The time of repetition was 4.5 s. Images were produced using a 32x32 mm field of view, 1-mm slice thickness, and 128x64 matrix. SWI used a specialized 3-dimensional gradient echo sequence with time of echo, 10 ms; time of repetition, 40 ms; flip angle of 15°, 32x32x24 mm3 field of view, 256x192x64 matrix, and flow compensation in all 3 directions. Diffusion-weighted imaging was acquired using a spin-echo sequence with pulsed diffusion-weighted gradients and one-shot echo-planar readout. The field of view was 32x32 mm2; 128x128 matrix, 1-mm slice thickness with 13 slices, time of repetition, 10 s and time of echo, 50 ms; 1 baseline of b=0 s/mm$^2$; 128 directions of diffusion gradients with b=1500 s/mm$^2$ for each slice. CE-T1WI was composed of 2 T1WI acquisitions, before and 6 minutes after injection of Gd-DTPA into a tail vein at a dose of 0.4 mL/kg. T1WI was acquired using a conventional multislice single spin-echo sequence with time of echo of 8 ms and time of repetition of 500 ms. The other MRI parameters in T1WI were the same as in T$_2$-weighted imaging.

Histological Staining

Rats were euthanized with ketamine (44 mg/kg IP) and xylazine (13 mg/kg IP). Brains were isolated, postfixed in 4% paraformaldehyde for 2 days at room temperature, and then processed for paraffin sectioning. Coronal sections (6-μm thick) were cut from each block and stained with hematoxylin and eosin (H&E) for the evaluation of ischemic lesion and blood in cerebral parenchymal tissue using light microscopy. Perls Prussian Blue (PPB) stain was performed for measurement of iron in cerebral parenchymal tissue, as evidence of hemorrhage. Double Bielschowsky’s silver and Luxol fast blue staining was performed for evaluation of axon and myelin.

Data and Statistical Analysis

Data analysis was performed in a blind fashion. MRI image analysis was generally performed with homemade software, Eigentool. T$_2$ maps were obtained pixel-by-pixel using a linear least-squares fit to the plot of the natural logarithm. SWI was analyzed using SPIN software. The multidirection diffusion-weighted imaging analysis was performed using Camine software. Fractional anisotropy (FA) map was derived from the multidirection diffusion-weighted imaging. Ischemic lesion volumes of MRI were measured on the T$_2$ maps. The mean plus 2× SD of the contralateral measurements on the T$_2$ maps was used as a threshold to identify lesion volume. To eliminate the influence of brain atrophy after stroke, the ischemic lesion volume were calculated and expressed as the ratio of lesion volume to contralateral hemispheric volume. The same measurement was performed on the subtraction image of pre-Gd T1WI from post-Gd T1WI. Because of hypointensity of hemorrhage on the SWI intensity image, the mean minus 2× SD of the contralateral measurements was used as threshold to identify HT after stroke. T1WI and SWI measurements were presented as the direct volumes. FA measurement was performed with 1 slice matched the histological section C for each animal. The difference of ischemic lesion areas obtained from the same slice of T$_2$ map between 1 day and 5 weeks after stroke was referred as the recovery region of interest. The FA map was first warped to the corresponding T$_2$ map; and then, the region of interests within the striatum were loaded onto the FA map for measurement.

The MicroComputer Imaging Device system (Imaging Research Inc, Ontario, Canada) was used for histological measurements. H&E- and PPB-stained sections were evaluated at x20 or x40 magnifications, respectively. With Bielschowsky’s silver and Luxol fast blue staining, 4 locations of the coronal section were used for axonal density quantification. The reactive areas of the recovery region of interest inside striatum were measured (percentage to field of view) under a x40 objective of optical microscope, using an average of all locations as the histological result.

ANOVA was performed. The effect was detected at the 0.05 level. Student t test was applied between animal groups of MRI measurements obtained at the same time points. MRI measurements are summarized as mean and SD.
Results

The ischemic lesion volumes quantitatively measured by $T_2$ maps, indicated in Figure 1A, do not exhibit any significant differences ($P>0.05$) during the period of 1 day to 5 weeks after stroke between the T2DM (33.8±15.3% at 5 weeks) and WT (28.7±16.3% at 5 weeks) rats. Representative MRI $T_2$ maps from 1 day to 5 weeks after stroke are shown in Figure 2 for T2DM and WT rats. With the edema declining with time after stroke, the volume of the ischemic lesion consequently experienced large changes from 1 day to 1 week after stroke for both T2DM and WT animals (Figure 1A). Lesion volumes from $T_2$ maps exhibited similar temporal profiles for both T2DM and WT rats (Figure 1A). The infarction volumes measured from the histological H&E coronal sections using the MicroComputer Imaging Device system were 37.9±10.4% of the contralateral hemisphere for the T2DM rats and 27.8±5.9% for the WT rats.

Unlike the lesion volumes, BBB disruption volumes of the T2DM rats measured with CE-T1WI of Gd-DTPA exhibited significant differences from the WT rats, as quantitatively demonstrated in Figure 1B. The volumes with Gd-DTPA enhancement acquired with CE-T1WI were significantly ($P<0.05$) larger in the T2DM rats than in the WT rats from 1 to 5 weeks after stroke. No evident Gd-DTPA leakage was found after that in the WT rat.

BBB disruption after ischemia may lead to HT. The hypointensity areas excluding veins in the SWI images are associated with hemorrhage. Quantitative SWI measurements of hemorrhagic volumes, as shown in Figure 1C, demonstrated that the T2DM rats exhibited significantly ($P<0.005$) larger hemorrhagic volumes ($\approx 10x$) than WT rats from 1 to 5 weeks after stroke. The representative T2DM and WT rats exhibited different evolutions of the HT after ischemia with SWI images in Figure 4. From the SWI images, areas of hypointensity which reflect hemorrhage, indicated by arrowheads in Figure 4, actively changed morphologically during 1 day to 5 weeks after stroke in the T2DM rat. Although hemorrhage spots identified in the WT rat changed little, where hemorrhage was detected starting at 1 week and no apparent change was subsequently found to 5 weeks after stroke. The hypointensity areas are much larger in the T2DM rat than in the WT rat.

Erythrocytes outside of blood vessels in the cerebral parenchymal tissue indicate hemorrhage. Analysis of H&E histological coronal sections under the light microscope showed that the red cells were diffusively present in the ischemic cerebral tissue in the T2DM rat, shown in Figure 5A, whereas the red blood cells were localized in a smaller region in the WT rat (Figure 5B). Prussian blue spots outside of blood vessels in the cerebral tissue in the T2DM rat, shown in Figure 5A, whereas the red blood cells were localized in a smaller region in the WT rat (Figure 5B). Prussian blue spots outside of blood vessels in the cerebral tissue in the T2DM rat, shown in Figure 5A, whereas the red blood cells were localized in a smaller region in the WT rat (Figure 5B).
brain parenchymal tissue were larger in size and much darker in color for the T2DM rat (Figure 5C) than that for the WT rat (Figure 5D). Combining the histological results of H&E and PPB staining for short- and long-term hemorrhage, respectively, the T2DM rat evidently had more severe and extensive hemorrhage after stroke than the WT rat, which coincided with the MRI measurements.

The temporal profiles of FA values of the recovery striatal tissue are demonstrated in Figure 1D. The FA values remained significantly ($P<0.02$) lower within 5 weeks after stroke for T2DM rats, compared with the WT group. The mean values of FA were $0.31\pm0.08$ for the WT group and $0.20\pm0.05$ for the T2DM group at 5 weeks after stroke. The axonal densities were histologically measured at 5 weeks after stroke on Bielschowsky’s silver and Luxol fast blue staining sections, as $24.1\pm7.7\%$ for the WT group versus $17.4\pm5.3\%$ for the T2DM group, which is significantly different ($P<0.05$) and consistent with FA measurements. However, the functional outcomes of the modified Neurological Severity Score$^a$ were marginally different ($P<0.06$) between the control ($5.89\pm0.60$) and T2DM ($6.89\pm1.27$) groups at 5 weeks after MCAo.

**Discussion**

Clinical data indicate that nondiabetic patients with transient hyperglycemia have smaller infarction volumes after stroke than diabetic patients.$^{3,4}$ Preclinical results on infarct size in diabetic and nondiabetic animals are equivocal. Pre-existing hyperglycemia significantly exacerbates the ischemic lesion volumes in most brain regions at 4 hours after stroke in adult Sprague–Dawley rats with a 2-hour/2-hour suture ischemia–reperfusion injury compared with normoglycemic cohorts.$^{20}$ However, compared with normoglycemia, the infarct volume was decreased in hypoglycemic rats and unaltered in acute diabetes mellitus induced by single streptozotocin injection 2 days before MCAo. With the T2DM model in Goto-Kakizaki rats, infarction volumes were significantly smaller and independent of the 3-hour/21-hour ischemia–reperfusion filament,$^9$ or 24-hour embolic MCAo models.$^1$ Conversely, infarction volumes were significantly increased after stroke in T2DM mice compared with nondiabetic mice.$^7,12$ Thus, there is a need to delineate the ischemic lesion volume for our animal model, a T2DM model induced by a single intraperitoneal injection of low-dose streptozotocin combined with a high-fat diet, and a 2-hour suture ischemia–reperfusion model in the young adult Wistar rat. Using dynamic $T_2$-weighted imaging measurements in the current study, shown in Figure 2A, the cerebral infarction volume of T2DM rats was not significantly different from that of nondiabetic controls from 1 day to 5 weeks after stroke. Histological measurements of infarction at 5 weeks after stroke present consistent results with MRI. This preclinical result, interestingly, coincides with the clinical outcome.$^{21}$

Unlike neuronal damage after stroke, more severe vascular damage has been consistently documented in patients with stroke and animals with diabetes mellitus.$^{3,4}$ However, in previous preclinical reports, BBB disruption and permeability rate in diabetic animals were regionally measured shortly after stroke from stained cerebral tissue sections, and measurements were limited to 1 time measurement using histological methods.$^{7,12}$ In contrast, using MRI in the present study, longitudinal measurements of BBB disruption were performed in stroke rats with or without T2DM from 1 day to 5 weeks after ischemia–reperfusion stroke. At 24 hours after stroke, T2DM rats had a marginally larger BBB disruption volume ($P<0.08$) than WT rats. The MRI data, as shown in Figure 2B, demonstrated that T2DM rats exhibited significantly larger volumes of BBB disruption, as indicated by measurement of Gd-DTPA leakage, starting from 1 week and persisted to 5 weeks after stroke ($P<0.005$), compared with WT rats. These data indicate that BBB disruption after stroke is a long-term problem in T2DM rats, which persists for ≥5 weeks after stroke. Vascular remodeling before MCAo in the T2DM rats because of hyperglycemia was not separately investigated in this study.

BBB disruption may lead to HT after ischemia. When blood cells leave a ruptured blood vessel, the erythrocytes die, and the hemoglobin of the cell is released into the extracellular space. With the hemoglobin losing oxygen, diamagnetic oxyhemoglobin becomes paramagnetic deoxyhemoglobin. Phagocytic cells engulf the hemoglobin to degrade it, producing hemosiderin, an iron-storage form, always found within macrophages and is especially
abundant in situations after hemorrhage, which can be identified histologically by Prussian blue stain, and importantly, by SWI.22

Because deoxyhemoglobin and hemosiderin present after hemorrhage, SWI is used to detect the HT after ischemia. As shown in Figure 2C, hemorrhagic volumes identified by SWI were significantly larger in T2DM rats than in WT rats from 1 day to 5 weeks after stroke (P < 0.05). Histological H&E and PPB staining pictures (Figure 5) support SWI results, which show increased hemoglobin (Figure 5A) for new hemorrhage and hemosiderin (Figure 5C) for old hemorrhage in the T2DM rat compared with the WT rat (Figure 5B and 5D), respectively.

MRI FA was able to monitor well-reorganized white matter, and FA has been used as an index of white matter recovery after treatment of stroke in rats.23 In the present study, the temporal profiles of FA measurements (Figure 1D) demonstrated a significant decrease (P < 0.02) in FA of the white matter in the recovery region of interest extending from the corpus callosum to the boundary of the stroke lesion in the T2DM rats, in contrast to the WT controls. These data suggest that T2DM may hamper the white matter reorganization involving the corpus callosum after stroke. The histological measurements with Bielschowsky’s silver and Luxol fast blue–stained sections parallel results as the MRI FA, that is, axonal density along the ischemic boundary in striatum was significantly lower (P < 0.05) in the T2DM rats than in WT rats at 5 weeks after stroke. Thus, in the present study, these FA and histological results on white matter may coincide with the increased functional deficits after stroke in diabetic patients and rats3,6 because white matter plays a pivotal role in neurological functions.

Conclusions
In the present study, measurements based on T1 maps demonstrate no significant difference of ischemic lesion volumes between T2DM and WT rats in 5 weeks after stroke, using a suture 2-hour occlusion and reperfusion stroke model and a low-dose streptozotocin injection combined with a high-fat food diet diabetic model of young adult Wistar rats. However, compared with WT rats, Gd-DTPA leakage measured by CE-T1WI indicates that T2DM rats suffered more severe BBB disruption from 1 to 5 weeks after stroke (P < 0.005), and SWI identified significant larger hemorrhagic volumes in T2DM rats throughout 5 weeks after stroke (P < 0.05). FA values of ischemic boundary in the striatum were consistently lower in the T2DM rats than in the WT controls, which suggest that T2DM hampers axonal density increase. MRI results were consistent with histological measurements.

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Disclosures
None.

References

Figure 5. With the histological hematoxylin–eosin (H&E) sections under light microscope, the red cells can be found diffusively in the ischemic cerebral tissue in the type 2 diabetes mellitus (T2DM) rat (everywhere in A, ×40), whereas the red cells appeared in a relative restrictive and smaller region in the wild-type (WT) rat (black arrow head in B, ×40). Perls Prussian Blue (PPB) stain demonstrated that the blue spots outside of vascular vessels in the brain parenchymal tissue for the T2DM rat (C, ×40) were more in number, larger in size, and darker in color than those for the WT rat (D, ×40). Bars in D, 40 μm.


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