Angiogenesis Detected After Embolic Stroke in Rat Brain Using Magnetic Resonance T2*WI

Guangliang Ding, PhD; Quan Jiang, PhD; Lian Li, PhD; Li Zhang, MD; Zheng Gang Zhang, PhD, MD; Karyn A. Ledbetter, BS; Lakshman Gollapalli, MS; Swayamparva Panda, MS; Qingjiang Li, MBA; James R. Ewing, PhD; Michael Chopp, PhD

Background and Purpose—This study uses T2* weighted imaging (T2*WI) to measure the temporal evolution of cerebral angiogenesis in rats subjected to embolic stroke up to 6 weeks after stroke onset with or without sildenafil treatment.

Method—Male Wistar rats were subjected to embolic stroke and treated with saline (n = 10) or with sildenafil (n = 11), with treatment initiated at 24 hours and continued daily for 7 days after onset of ischemia. T2*WI measurements were performed at 24 hours after embolization and weekly up to 6 weeks using a 7-Tesla system. Histological measurements were obtained at 6 weeks after MRI scans.

Results—Using T2*WI, cerebral angiogenesis was detected starting from 4 weeks and from 2 weeks after onset of embolic stroke in saline and sildenafil treated rats, respectively. Significant differences in the temporal and spatial features of angiogenesis after embolic stroke up to 6 weeks after onset of stroke were found between saline and sildenafil treated rats and were identified with T2*WI. MRI permeability parameter, Ki, complementarily detected angiogenesis after ischemia in embolic stroke rats. Sildenafil treatment of stroke rats significantly enhanced the angiogenesis, as confirmed histologically.

Conclusions—T2*WI can quantitatively measure the temporal evolution of angiogenesis in rats subjected to embolic stroke. Compared to control rats, sildenafil treatment significantly increased angiogenesis in treated animals up to 6 weeks after stroke. (Stroke. 2008;39:1563-1568.)

Key Words: angiogenesis ■ embolic stroke ■ magnetic resonance imaging ■ sildenafil ■ T2* weighted imaging

Magnetic resonance imaging (MRI) can noninvasively assess the evolution of several indices that characterize cerebral tissue after stroke with or without treatment. Conventional MRI can precisely track the lesion volume and provides complementary information about the status of cerebral tissue after embolic stroke.2–4 Because susceptibility-weighted imaging (SWI) is sensitive to venous blood vessel volume,5 SWI may identify angiogenesis in the stroke brain after the newly-generated cerebral vasculature has matured.6 Based on the same underlying principle as SWI, T2*WI weighted imaging (T2*WI) may detect angiogenic tissue containing newly developed mature vasculature. T2*WI also identifies hemorrhage,7–8 and it may distinguish between angiogenesis and hemorrhage after stroke. Hence, angiogenesis in brain after stroke may be dynamically and noninvasively investigated using T2*WI.

Sildenafil is an inhibitor of the phosphodiesterase type 5 (PDE5) enzyme which is highly specific for hydrolysis of cyclic guanosine monophosphate (cGMP).9–10 Administration of sildenafil, therefore, by inhibiting cGMP breakdown, causes intracellular accumulation and increased brain levels of cGMP.11–13 Previous studies showed that treatment of rats with sildenafil significantly increased the cortical levels of cGMP in both hemispheres for nonischemic rats,12 and in the ipsilateral hemisphere at 7 days after stroke compared with levels in control animals.13 Elevated cGMP levels in cerebral tissues may be involved in promoting angiogenesis during recovery up to 4 weeks after embolic stroke in rats.12–13 However, additional studies, especially noninvasive measurements which can be applied clinically, are needed to investigate the relationship between the sildenafil treatment of stroke and angiogenesis. Here, for the first time, we used T2*WI to quantitatively investigate cerebral angiogenesis up to 6 weeks after stroke in rats subjected to embolic stroke with and without sildenafil treatment. Our data demonstrate that T2*WI identifies cerebral angiogenesis after embolic stroke in rats, and sildenafil treatment enhances angiogenesis which occurs earlier in sildenafil treated rats than in the saline treated rats.

Materials and Methods
All studies were performed in accordance with institutional guidelines for animal research under a protocol approved by the IACUC of Henry Ford Hospital.
Animal Model and Experimental Protocol
Male Wistar adult rats at ages of 8 to 12 weeks, weighing 300 to 350 g, were subjected to embolic stroke by placement of an aged white clot at the origin of the middle cerebral artery (MCA). Rats with embolic stroke were randomly assigned to treatment (n=11) and control (n=10) groups. Sildenafil (Viagra, Pfizer Inc) was administered subcutaneously at a dose of 10 mg/kg to rats in the treated group 24 hours after MCA occlusion and daily for an additional 6 days. The selected dose has been previously shown to be effective for this model. Rats in the control group were treated with the same volume of saline as in the treated group. All rats were euthanized 6 weeks after stroke.

Magnetic Resonance Imaging Measurements
MRI measurements were performed using a 7 Tesla system with Bruker console (Bruker-Biospin Inc). During MRI measurements, anesthesia was maintained using a gas mixture of nitrous oxide (70%), oxygen (30%), and halothane (0.75 to 1.00%). Rectal temperature was kept at 37°C±1.0°C using a feedback controlled water bath. A tripilot imaging sequence was used for reproducible positioning of the animal in the magnet at each MRI session. T2*WI, SWI, and delayed blood-tissue transfer constant (Kt) of diethyleneetriamine pentaacetic acid (Gd-DTPA) measurement were performed before ischemia, repeatedly at 24 hours, and weekly up to 6 weeks after embolization for all animals. These sequences required approximately 1 hour of scan time.

Histological Immunohistochemistry
Animals were anesthetized with ketamine (44 mg/kg i.p.) and xylazine (13 mg/kg i.p.), and were transcardially perfused with a different temporal profile and morphology, hemorrhagic transformation (HT) was distinguished from angiogenesis by calculating the mean value minus three times the standard deviation (SD) of the contralateral tissue. SWI was analyzed using SPIN software. This software allows the choice of several algorithms to remove background field effects and focus on the local field changes. The phase information was extracted from phase maps and used as a filter to enhance the local field changes reflected in usual modular images.

Results
T2*WI images were reconstructed using a 128×128 matrix. The T2* maps were produced using a linear least-squares fit to the plot of the natural logarithm of normalized image intensity of T2*WI versus TE values. The areas throughout the ipsilateral hemisphere with low T2* values on T2* maps were identified as related to angiogenesis by calculating the mean value minus three times the standard deviation (SD) of the contralateral tissue. SWI was analyzed using SPIN software. This software allows the choice of several algorithms to remove background field effects and focus on the local field changes. The phase information was extracted from phase maps and used as a filter to enhance the local field changes reflected in usual modular images.

All 11 T1 maps acquired by Look-Locker presented time-dependent changes in T1, and were used to form a Kt map by using Palak matrix analysis of compartmental dynamics. Measurements are summarized as mean value with SD. The group averages are presented as mean value with standard error (SE). The differences of MRI and histological measurements between 2 groups were analyzed by t-test. The significance level (α) was set at 5%.
Quantitative T2* characterization of angiogenesis after stroke up to 6 weeks is presented in Figure 2. The areas throughout the ipsilateral hemisphere with low T2* values on T2* maps were identified as related to angiogenesis by the mean value minus 3 times the SD of the contralateral tissue. Two weeks after onset of stroke, the angiogenic area (Figure 2a) identified with low T2* values in sildenafil treated animals rapidly increased and achieved a maximum size (in pixels: 1 pixel = 0.0625 mm2) at 2 weeks, and remained at maximum up to 5 weeks after stroke. In contrast, the size in control animals gradually increased within 6 weeks after stroke. The angiogenic areas were significantly different between the treated and control groups from 1 week to 4 weeks after stroke with \( P < 0.05 \) (\( P < 0.01 \) at 1 to 3 weeks).

Figure 2b shows the mean values of T2* in these angiogenic regions. The T2* mean values related to angiogenesis in sildenafil treated animals were minimum at 3 weeks after stroke, while the T2* values in control animals gradually decreased during the 6 weeks after stroke. The averaged T2* values related to angiogenesis exhibit significant differences between control and treated rats from 1 week to 4 weeks after stroke with \( P < 0.05 \) (\( P < 0.01 \) at 1 to 3 weeks).

Supportive SWI data from the same representative control and treated rats above with the T2* maps are shown in Figure 3. There are 2 sets of images of axial and coronal sections for each control and treated rats. The low intensity area identified by white arrows in SWI images were clearly present at 3 or 5 weeks from axial or coronal sections (as averaged at 4 weeks) after stroke for the control animal (1st and 3rd rows), and at 2 weeks after stroke for the treated rat in both axial and coronal sections (2nd and 4th rows), respectively.

MRI data for detecting angiogenesis in this study were confirmed by histological measurements at 6 weeks after stroke using EBA staining, as shown in Figure 4. An EBA stained slice (Figure 4A) of the treated rat demonstrates angiogenesis enhanced by sildenafil treatment, as shown in an enlarged image with red arrows (Figure 4B). The T2* map (Figure 4D) and SWI image (Figure 4E) obtained 6 weeks after stroke detected the angiogenic area (red arrows). This area closely matched the histological result compared with a warped image (Figure 4C), which was warped from the actual EBA stained slice (Figure 4A), referred to a T2WI image.
Angiogenesis after embolic stroke was detected by SWI. For a control rat treated with saline, SWI images exhibited evidence of angiogenesis (white arrow) started from 3 weeks in a transverse section (the 1st row) and 5 weeks in a coronal section (the 3rd row) after stroke. For a sildenafil-treated animal, angiogenesis identified by SWI (white arrow) occurred at 1 week after stroke in both axial (the 2nd row) and coronal (the 4th rows) sections. The transverse images were reconstructed from the original coronal images.

**Discussion**

The present study demonstrates, for the first time by using T2*WI, that cerebral angiogenesis after embolic stroke in rats can be quantitatively and dynamically investigated. The differences of angiogenesis between control and treated animals can be identified by T2*WI. In contrast to embolic stroke rats in the saline-treated group, angiogenesis detected by T2*WI (as well as SWI) was significantly accelerated after stroke in sildenafil-treated animals, where the sildenafil treatment was initiated 24 hours after stroke for 7 consecutive days. Histological data demonstrate that the sildenafil treatment significantly enhances cerebral angiogenesis in rats at 6 weeks after stroke. A previous report from the same animals demonstrated that the increased angiogenesis in sildenafil-treated animals with stroke correlates with elevated local CBF in the ischemic boundary area and appears to drive neurological functional recovery.16

Because SWI uses susceptibility differences between cerebral tissues, it is sensitive to cerebral veins because of blood oxygenation level dependent (BOLD) effects.5–6 SWI image intensity is reduced in the area where the newly-generated veins assume function as angiogenesis matures. Comparing SWI images of the treated animal (the 2nd and 4th rows in Figure 3) with control images (the 1st and 3rd rows in Figure 3), angiogenesis began to mature from 2 weeks after stroke in sildenafil treated rats. In these rats, angiogenesis was accelerated compared to control rats, where the angiogenesis began to mature from 4 weeks after stroke. These data indicate that the sildenafil treatment of embolic stroke in rats evokes early and rapid angiogenesis after embolism.

T2* imaging has been used to detect HT after ischemic stroke.7–8 The image contrast mechanism of the T2* map is attributed to the local magnetic field inhomogeneities caused by tissue susceptibility differences, similar to SWI, in addition to T2 relaxation. Thus, we expect that the T2* map, as SWI, may detect angiogenesis after stroke. This study demonstrated that T2* maps not only can detect angiogenesis after stroke in both control and treated rats, but can also distinguish the different temporal profiles of angiogenesis between control and treated animals. By measuring the temporal profiles of quantitative T2* values, T2* maps clearly identified angiogenesis areas in all animals at 6 weeks after stroke, as confirmed by histological measurements. For sildenafil treated rats, the quantitative T2* measurements revealed that the average size of angiogenic areas approached its maximum at 2 weeks after stroke and T2* mean value of the areas approached its minimum at 3 weeks after stroke, respectively. However, for saline-treated rats, the values did not reach the maximum or minimum before 6 weeks after stroke (Figure 2a and 2b). Thus, in addition to providing information on the evolution of angiogenic areas as SWI does, T2*WI can also provide the temporal profiles of T2* values. The quantitative T2* values may permit calculation of vascular density.20

Hemorrhage generally occurs within the ischemic core, where the ischemia is most severe, and angiogenesis primarily occurs along ischemic boundary region.13,21 HT after ischemia may be identified and distinguished from angiogenesis by comparing the differences in shapes and temporal profiles of decreased T2*. Most BBB damage after embolic stroke in the rat usually occurs before 48 hours after stroke.21 On T2* maps, HT, if present, is
revealed by a hypointensity area from 1 to 6 weeks after stroke (the 3rd row T2* maps in Figure 1). Once the HT was detected in the T2* map at 1 week after stroke, the size and shape of the HT region generally remained unchanged (Figure 1), which was the major difference from the area identified as angiogenic. The shape of HT detected by T2* was round-like, which differed from the curve-like geometry of angiogenic areas. SWI provided the same information on location and size of HT at 1 to 6 weeks after stroke as T2*. However, SWI shows an improved ability to detect HT area at an earlier stage of stroke, as shown in Figure 4M and 4N. Because of edema after ischemia, T2* values were highly and largely elevated in the ipsilateral hemisphere, which impaired the ability of the T2* map to detect HT within this area. When SWI detected the HT at 1 day after ischemia (Figure 4M), the T2* did not detect the HT until at 2 days after onset of ischemia (Figure 4N).

We note that T2*WI does not specifically identify angiogenesis, with hemorrhage also associated with T2*WI hypointensity. However, angiogenesis and HT can be distinguished based on: (1) different evolution patterns with T2* and Ki maps, (2) different shapes on T2* maps (curve-like versus round-like), (3) different location (boundary versus core). Thus, T2*WI may find application is identifying angiogenesis and hemorrhage, and this method warrants further investigation and possibly clinical application.

The MRI Ki map is a blood-to-brain permeability index and is sensitive to Gd-DTPA leakage attributable to open (damaged or undeveloped) BBB in vasculature. The BBB of newly-generated vessels during angiogenesis are not mature. As a result, K is a good index to monitor angiogenesis after stroke. Elevated K values were detected as 2 developing patterns after stroke up to 6 weeks. The first pattern showed a temporal profile of elevated K from 1 to 3 weeks after stroke in sildenafil-treated animals (the 2nd row of K maps in Figure 1), which suggests that angiogenesis is present during this period. This pattern was similar to the results...
obtained in stroke rats with cell therapy. In the stroke rats treated with neural progenitor cells, the transplanted cells arrive at their destination within 1 week after grafting and angiogenesis activity occurs in sites either adjacent to or surrounded by the grafted cells. In this situation, it is difficult to use the T₂* to detect angiogenesis because the grafted cells are labeled with iron. While the other pattern, for control animals treated with saline, demonstrated that the regional Kᵥ values remained elevated from 1 to 6 weeks after stroke, as shown in the first row of Kᵥ maps in Figure 1. This indicates that angiogenesis is slowly and continuously developing from 1 week up to 6 weeks after onset of stroke. Thus, therapies, such as pharmacological (sildenafil) and cell therapies, may shorten the time window of angiogenesis, by accelerating its development. BBB damage or HT after embolic stroke was detected with Gd-DTPA enhancement at 24 hours in the Kᵥ map, indicating that angiogenesis is slowly and continuously developing and BBB disruption after embolic stroke.

Sildenafil citrate is a potent inhibitor of PDE5 and causes intracellular accumulation of cGMP. cGMP signaling in the central nervous system promotes angiogenesis, neurogenesis, and axonal outgrowth in the adult rats. Previous non-MRI studies revealed that treatment of embolic stroke in rats with sildenafil significantly enhances angiogenesis after stroke. In the present study, we have used T₂* maps to quantitatively, noninvasively, and dynamically investigate angiogenesis after embolic stroke in the rat. Angiogenesis was present after stroke in both saline- and sildenafil-treated groups, as indicated by T₂* maps and SWI images, and vascular density significantly increased in the sildenafil-treated group compared to saline-treated animals as revealed by histological measurements (Figure 4). The histological sections were deformed after processing, because the cerebral tissue of the ischemic core was severely damaged at 6 weeks after stroke. This reduced our ability to match the angiogenesis area detected by T₂* with histology. However, we were still able to match select sections after warping (Figure 4C through 4E). These histological results were consistent with results in previous non-MRI studies.

In summary, our data in this study demonstrated that T₂*WI can be used to detect and identify not only hemorrhage after ischemia, but also angiogenesis after stroke in treated and control animals. The T₂* maps provide quantitative data that distinguish angiogenic profiles after treatment of embolic stroke in rats with sildenafil from control stroke animals.

Sources of Funding
This work was supported by NINDS grants PO1 NS23393, PO1 NS42345, RO1 NS38292, RO1 NS48349, RO1 NS43324, and HLI64766.

Disclosures
None.

References
Angiogenesis Detected After Embolic Stroke in Rat Brain Using Magnetic Resonance T2*WI

Guangliang Ding, Quan Jiang, Lian Li, Li Zhang, Zheng Gang Zhang, Karyn A. Ledbetter, Lakshman Gollapalli, Swayamprava Panda, Qingjiang Li, James R. Ewing and Michael Chopp

Stroke. 2008;39:1563-1568; originally published online March 20, 2008; doi: 10.1161/STROKEAHA.107.502146

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/39/5/1563

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/