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

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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. Because susceptibility-weighted imaging (SWI) is sensitive to venous blood vessel volume, SWI may identify angiogenesis in the stroke brain after the newly-generated cerebral vasculature has matured. Based on the same underlying principle as SWI, T2* weighted imaging (T2*WI) may detect angiogenic tissue containing newly developed mature vasculature. T2*WI also identifies hemorrhage 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). Administration of sildenafil, therefore, by inhibiting cGMP breakdown, causes intracellular accumulation and increased brain levels of cGMP. Previous studies showed that treatment of rats with sildenafil significantly increased the cortical levels of cGMP in both hemispheres for nonischemic rats, and in the ipsilateral hemisphere at 7 days after stroke compared with levels in control animals. Elevated cGMP levels in cerebral tissues may be involved in promoting angiogenesis during recovery up to 4 weeks after embolic stroke in rats.

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 a 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 T1WI were acquired with the spin echo technique. A tripilot imaging sequence was used for reproducible positioning of the animal in the magnet at each MRI session. T2*WI, SWI, and T1WI were acquired with the spin echo technique. In combination with T2*WI, SWI, and T1WI, a 3D gradient echo T2* image was obtained 30 minutes after the administration of contrast material. This acquisition was 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 heparinized saline followed by 10% neutral buffered formalin. The brain was removed and immersed in formalin solution for 1 hour, after which a total of 7 2-mm-thick blocks of brain tissue were cut, processed, and embedded in paraffin.

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MCID image analysis system (Imaging Research Inc) with a 40× objective (Olympus BX40) and a 3-CCD color video camera (Sony DXYC-907MD) was used for histological immunohistochemical measurements. Coronal sections (6 μm thick) were cut from each block and stained with hematoxylin and eosin (H&E) for the evaluation of cerebral tissue. The Ki maps in Figure 1 exhibit typical Ki evolution patterns 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 T1 maps acquired by Look-Locker presented time-dependent changes in T1, and were used to form a Kt map by using Patlak 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%.

Results

T2* maps detected evidence of angiogenesis after stroke in both saline-treated (control, C) and sildenafil-treated (treated, T) animals. The mean value of T2* of normal cerebral tissue is 38.5±6.2 milliseconds. Figure 1 shows the evolution of an axial section of T2* maps from a control rat (1st row) and a treated animal (2nd row). The T2* maps of the control rat demonstrate that the angiogenesis represented as the low intensity areas excludes corpus callosum on T2* maps first appeared at 4 weeks (red arrow), whereas the T2* maps of the sildenafil treated rat showed the angiogenic low intensity areas on T2* maps from 2 weeks (red arrow), respectively, after stroke. Low intensity areas along ischemic boundary in T2* maps indicate the formation of venous vasculature attributable to angiogenesis. Before these characteristic low-intensity areas of angiogenesis formed, low-intensity areas in T2* maps had appeared at 2 and 3 weeks in the control rat, and at 1 week for the sildenafil-treated rat, respectively, after stroke, as indicated by white arrows. These areas, however, were not identified as angiogenic because their T2* values were within thresholds (mean, 3*SDs) of the images, though they may indicate the onset of angiogenesis after stroke. With a different temporal profile and morphology, hemorrhagic transformation (HT) was distinguished from angiogenesis by T2* maps, as shown in the third row of T2* images of Figure 1. The lower T2* value region caused by HT generally appeared on the T2* map at 1 week and up to 6 weeks after stroke. The shape and size of the region identified as HT by T2* exhibited little change up to 6 weeks after stroke, and was quite different from angiogenesis. The Ki maps in Figure 1 exhibit typical Kt evolution patterns of angiogenesis for the same control and treated rats. For the control rat (1st row, C), regional Kt values were elevated after 1 week (white arrow), and the peak period was between 2 to 5 weeks after stroke (red arrow). In the treated rat (2nd row, T), regional Kt increased from 1 week (red arrow) to 3 weeks after stroke, and then regressed toward normal from 4 weeks after stroke. The elevated Kt value region caused by the blood-brain-barrier (BBB) disruption (3rd row, HT) appeared at 1 day after stroke on the Kt map (red arrow) and then disappeared starting from 1 week after stroke. The averaged Kt value of normal cerebral tissue is 3.13±2.43/min.
Quantitative $T_2^*$ characterization of angiogenesis after stroke up to 6 weeks is presented in Figure 2. The areas throughout the ipsilateral hemisphere with low $T_2^*$ values on $T_2^*$ 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 $T_2^*$ values in sildenafil treated animals rapidly increased and achieved a maximum size (in pixels: 1 pixel = 0.0625 mm$^2$) 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 $T_2^*$ in these angiogenic regions. The $T_2^*$ mean values related to angiogenesis in sildenafil treated animals were minimum at 3 weeks after stroke, while the $T_2^*$ values in control animals gradually decreased during the 6 weeks after stroke. The averaged $T_2^*$ 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 $T_2^*$ 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 $T_2^*$ 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.
(Figure 4F), in this animal. Figure 4G through 4I shows representative EBA pictures (40× microscope) for ipsilateral slices of control and sildenafil treated rats, and the contralateral slice of a sildenafil-treated rat, respectively. For both control (Figure 4G) and treated (Figure 4H) rats with embolic stroke, ipsilateral vessels were enlarged and vascular densities were increased in angiogenic areas after stroke compared to those in the homologous area of the contralateral hemisphere (Figure 4I). Quantitatively, the vascular density of the angiogenesis area indicated by MRI images along the ischemic boundary area in the ipsilateral hemisphere increased 53.8±21.1% for the treated group and 28.0±14.8% for the control animals (Figure 4J) compared to the corresponding homologous region vascular density (with an average number of 366±23 vessels/mm²) in the contralateral hemisphere at 6 weeks after stroke. Vascular density, hence, angiogenesis was significantly enhanced in the sildenafil treated rats compared with saline treated rats (P<0.03).

An additional rat with hemorrhage, confirmed histologically at 48 hours after stroke by H&E staining (red arrow in Figure 4K and Figure 4L, 20× microscope magnification), demonstrates the ability of the T²* map and the SWI to detect hemorrhagic transformation after stroke. At 24 and 48 hours after embolic stroke, the SWI image and T²* map identified the HT, as shown in Figure 4M and 4N with red arrow heads, respectively.

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.5–8 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.14 The BBB of newly-generated vessels during angiogenesis are not mature. As a result, Ki is a good index to monitor angiogenesis after stroke. Elevated Ki values were detected as 2 developing patterns after stroke up to 6 weeks. The first pattern showed a temporal profile of elevated Ki from 1 to 3 weeks after stroke in sildenafil-treated animals (the 2nd row of Ki maps in Figure 1), which suggests that the angiogenesis is present during this period. This pattern was similar to the results

Figure 4. Histological measurements by using EBA staining showed angiogenesis for a treated rat (A) indicated by the red arrows in the enlarged picture under 10× microscopy (B). The processed image (C) of the real picture (A), warped to a T2WI image (F), matched the results detected by T2* map (D) and SWI image (E) at 6 weeks after stroke. Blood vessels were enlarged and vascular densities were higher in angiogenesis areas for both control (G) and treated (H) animals measured under 40× microscope, compared to those in corresponding areas of the contralateral hemisphere (I). Quantitatively, the angiogenesis was significantly enhanced by the sildenafil treatment in embolic rats compared with control rats treated with saline ($P<0.03$) (J). An embolic stroke rat with hemorrhage (red arrow) at 48 hours after stroke confirmed by H&E stained slice (K) and 20× microscope enlarged picture (L) was used to verify T2* map and SWI image for detecting HT after stroke. SWI image (M) and T2* map (N) detected the HT (red arrows) at 24 and 48 hours after embolic stroke, respectively. Bars=100 μm.
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 K values remained elevated from 1 to 6 weeks after stroke, as shown in the first row of K 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 K maps and no enhancement in K maps from 1 to 6 weeks after stroke (the 3rd row of K maps in Figure 1). Thus, K maps complementarily provide data with T2* maps to identify both angiogenesis 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 from control stroke animals.

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**Disclosures**

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