Longitudinal Magnetic Resonance Imaging of Sildenafil Treatment of Embolic Stroke in Aged Rats

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Background and Purpose—Sildenafil provides restorative therapeutic benefits in the treatment of experimental stroke. The majority of experimental studies on treatment of stroke have been performed in young animals; however, stroke is primarily a disease of the aged. Thus, using MRI, we evaluated the effects of sildenafil treatment of embolic stroke in aged animals.

Methods—Aged male Wistar rats (18 months) were subjected to embolic stroke and treated daily with saline (n=110) or with sildenafil (n=110) initiated at 24 hours and subsequently for 7 days after onset of ischemia. MRI measurements were performed at 24 hours and weekly to 6 weeks after embolization.

Results—MRI and histological measurements demonstrated that sildenafil treatment of aged rats significantly enhanced angiogenesis and axonal remodeling after stroke compared to saline-treated aged rats. Local cerebral blood flow in the angiogenic area was elevated and expansion of the ipsilateral ventricle and, consequently, brain atrophy was significantly reduced in the sildenafil-treated rats.

Conclusions—Treatment of embolic stroke in aged rats with sildenafil significantly augments angiogenesis and axonal remodeling, which increased local blood flow and reduced expansion of the ipsilateral ventricle 6 weeks after stroke compared to control aged rats. MRI can be used to investigate brain repair after stroke in aged rats. (Stroke. 2011;42:3537-3541.)

Key Words: aged rat ■ embolic stroke ■ neurorestorative treatment ■ sildenafil

Advanced age is an important risk factor for stroke and a predictor of poorer outcome after stroke in elderly patients compared with younger patients.1 During aging, many physiological and pathophysiological functions are altered. Aged rats exhibit marked decreases in brain proteasomal activity compared with young rats after induction of intermittent hypoxia, which is associated with greater impairment of spatial learning.2 Aged rats subjected to stroke have a higher mortality rate and worse neurological deficits than young rats, and pharmacological effects after treatment of stroke in young rats may not necessarily translate to older rats.3–5 The majority of experimental studies of stroke have been performed in young animals, and the failure to translate laboratory data showing therapeutic benefit of treatment to the stroke patient may, in part, be attributed to the failure to perform preclinical studies in aged animals.

By inhibiting cyclic guanosine monophosphate break-down,6 sildenafil, a phosphodiesterase type 5 inhibitor, causes intracellular accumulation and increased brain levels of cyclic guanosine monophosphate.7 Administration of sildenafil significantly increases the cortical levels of cyclic guanosine monophosphate in both hemispheres for nonischemic young rats7 and in the ipsilateral hemisphere at 7 days after stroke for both ischemia-insulted young and aged rats compared with levels in nontreated stroke animals.3 Elevated cyclic guanosine monophosphate levels in cerebral tissues may promote angiogenesis during recovery up to 4 weeks after embolic stroke in both young and aged rats.3 However, aged rats exhibit a significant reduction of vascular density and impairment of functional recovery after stroke compared with young rats, and age was also associated with a reduction of elevated cyclic guanosine monophosphate levels after treatment of stroke with sildenafil in rats.3 The primary effect of sildenafil is vasorelaxing, which may cause a decrease of blood pressure. However, the dose of sildenafil administered in the current study did not cause the blood pressure to be outside the normal biophysical ranges.

Using MRI, angiogenic cerebral tissue after ischemic stroke in young rats can be identified by T2*-weighted imaging with or without sildenafil treatment.8 Accordingly, elevated regional cerebral blood flow has been measured in the ischemic boundary zone using perfusion-weighted imaging with continuous arterial spin labeling.9 Diffusion anisotropy (DA) derived from diffusion-weighted imaging in 3 mutual perpendicular directions10 provides a means for delineating the anatomic connectivity of white matter11 and was
used to detect pathological tract disruption and remodeling after stroke in adult rats. A recent MRI study demonstrated that after stroke, the volume of the ipsilateral ventricle measured by T2-weighted imaging is a sensitive index of cerebral tissue loss (brain atrophy) and repair in young rats with or without erythropoietin treatment. However, whether MRI can identify brain remodeling after stroke in aged rats with or without a restorative treatment remains unknown because of the decreased recovery ability, worse stroke deficits, and significant reduction of therapeutic response to treatment after stroke in aged rats.

Therefore, we tested the hypothesis that MRI also can detect enhanced neurorestorative processes after stroke in aged rats treated with sildenafil compared with saline treatment.

**Materials and Methods**

**Animal Model and Experimental Protocol**

All studies were performed in accordance with institutional guidelines for animal research under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Henry Ford Hospital. Male Wistar rats (Jackson Laboratory, Bar Harbor, ME) 18 months of age and weighing ~560 g were subjected to embolic stroke and randomly assigned to either the treatment (n=10) or control groups (n=10). The model of embolic stroke, briefly, uses a 4-cm-long aged clot slowly injected into the internal carotid artery to the origin of the middle cerebral artery. In the treatment group, sildenafil (Viagra; Pfizer) was administered subcutaneously at a dose of 10 mg/kg daily for 7 days starting 24 hours after middle cerebral artery occlusion. The selected dose has been previously shown to be effective for this model. The control group received an equal volume of saline.

MRI and functional tests (including adhesive removal test, foot-fault test, and a modified neurological severity score) were performed 24 hours and weekly to 6 weeks after stroke for all rats in a double-blind fashion. All animals were euthanized 6 weeks after stroke.

**MRI Measurements**

MRI measurements were performed using a 7-T Bruker system (Bruker-Biospin). During MRI measurements, anesthesia was maintained using a gas mixture of N2O (70%), O2 (30%), and isoflurane (1.00%–1.50%). Rectal temperature was kept at 37°C ± 1°C using a controlled water bath.

A tripilot sequence was used for reproducible positioning of the animal in the magnet at each MRI session. MRI measurements (including T2-weighted imaging for ischemic lesion and ventricular volumes, T2*–weighted imaging for angiogenesis, diffusion-weighted imaging for axonal remodeling, and perfusion-weighted imaging for CBF) were performed, as previously described.

**Histology**

Animals were anesthetized with ketamine (44 mg/kg intraperitoneal) and xylazine (13 mg/kg intraperitoneal) and transcervically perfused with heparinized saline, followed by 10% neutral-buffered formalin. The brain was immersed in 4% paraformaldehyde in phosphate-buffered saline at 4°C overnight, and seven 2-mm-thick blocks of brain tissue were cut, processed, and embedded in paraffin.

The MicroComputer Imaging Device system (Imaging Research) was used with a 40× objective (Olympus BX40) and a 3-Charge-Coupled Device (CCD) color video camera (Sony DXC-970MD) for histological measurements. Coronal 6-μm-thick sections were stained with hematoxylin and eosin to evaluate cerebral infarction, with endothelial barrier antigen to quantify cerebral vessels, and with Bielschowsky silver and Luxol fast blue to assess myelinated axons. Four fields of view in each coronal section were used for quantification.

**Data and Statistical Analysis**

MRI images acquired at various times and histological section images were reconstructed, coregistered, and analyzed using a homemade software package, Eigentool. The difference of the ischemic lesion sizes in T2 maps acquired at 24 hours (Figure 1A) and at 6 weeks (Figure 1B) after stroke are referred to as the recovery area (Figure 1C), T2*, CBF, and DA values of recovery ischemic tissue and homologous tissue mirrored in contralateral hemisphere were also measured and were used to obtain ratios.

Ventricular or ischemic lesion volumes were determined by T2 maps were acquired after stroke using values above the mean plus 2 SD of the contralateral measurements. The total volumes were the sum of the volumes in the 5 central slices.

MRI measurements are summarized as mean value with SD. Differences in the MRI data between groups were analyzed by a mixed model of ANOVA and ANCOVA. For the longitudinal MRI measurements, the analysis started testing the group and time (without baseline time point) interaction, followed by testing the group difference at each time point if the interaction or overall group effect was detected at the 0.05 level.

**Results**

Stroke was induced in 48 aged rats, 26 of whom died (19 within 24 hours, 6 between 24 hours and 48 hours, and 1 at 1 week), yielding a mortality rate of 54%. The primary reason for mortality was attributable to stroke complication. Two rats were excluded from the study because the T2 maps acquired at 24 hours after stroke showed no ischemic lesion. Ischemic lesion volumes using hematoxylin and eosin sections were measured as 26.6% ± 9.4% of ipsilateral hemisphere for the sildenafil treated rats and 26.7% ± 7.3% for the control rats 6 weeks after stroke. No differences were found for the lesion volumes between the treated and control groups of the aged rats (P>0.9).

T2* maps detected low-intensity regions after stroke along the ischemic boundary in both control (treated with saline) and sildenafil-treated aged rats. On a typical T2* map obtained 4 weeks after stroke from a representative aged rat treated with sildenafil whose ischemic lesion area was demarcated by hyperintensity on a T2 map acquired 24 hours after stroke (Figure 1A), hypointensity regions were apparent (white arrows in Figure 2A). One week later, the CBF map obtained from the same rat revealed elevation of CBF values.
(indicated by arrows in Figure 2B) in the areas of hypointensity measured on the T2* map. Six weeks after stroke (2 weeks after the identification of hypointensity region on the T2* map), the area with elevated CBF exhibited increases of DA values analyzed by diffusion measurements (identified by arrows in Figure 2C). The ischemic lesion size and expansion of the ipsilateral ventricle after stroke in this treated rat were demonstrated on the T2 maps acquired 6 weeks after middle cerebral artery occlusion (Figure 1B). In contrast, for the control aged rats, the hypointensity region on T2* map and hyperintensity areas on CBF and DA maps, respectively, were much less obvious. Quantitative MRI measurements were then used to analyze temporal changes in aged rats after stroke.

Quantitative longitudinal MRI measurements demonstrated temporal features of T2*, CBF, and DA for restorative cerebral tissue after stroke with or without sildenafil treatment in the aged rats. Compared to the control aged rats, T2* ratios (Figure 3A) of cerebral tissue along the ischemic boundary zone in the sildenafil-treated group had consistently lower values during the 6 weeks after stroke. After the initial decrease, however, T2* ratios increased starting from 5 weeks in the treated group. T2* ratios monotonically decreased in the control group to 6 weeks after stroke.

Higher CBF ratios were observed in sildenafil-treated aged animals during the experiments (24 hours to 6 weeks after stroke) in contrast to control aged rats (Figure 3B). DA values monotonically increased after stroke in the sildenafil-treated group but were delayed by 1 week in controls (Figure 3C). Higher DA ratios were measured in treated rats than in control rats during the experiments.

Temporal changes of ventricular volume ratios (ipsilateral versus contralateral) for both treated and control groups of aged rats are shown in Figure 3D. Ventricular volume ratios monotonically increased after stroke for all aged rats (D). However, the rate of increase was slower in the treated group from 1 week after stroke.

**Figure 2.** For a representative aged rat with sildenafil treatment after ischemia, the hypointensity regions in T2* map (A) were obtained 4 weeks after stroke. The elevated cerebral blood flow (CBF) was observed 1 week later (B). Six weeks after stroke, an increase of diffusion anisotropy (DA) values was apparent (C).

**Figure 3.** T2* ratios (A) of cerebral tissue in the sildenafil-treated group had lower values during 6 weeks after stroke than in the control group; the differences were significant at 4 weeks and 5 weeks after stroke \(P < 0.04\). Higher cerebral blood flow (CBF) ratios were observed in sildenafil-treated aged animals compared to control rats (B). At 6 weeks after stroke, CBF was significantly higher in treated group than in the control group \(P < 0.05\). Diffusion anisotropy (DA) values monotonically increased after stroke in aged rats (C). Starting from 4 weeks after stroke, the DA ratios were significantly different between the 2 groups \(P < 0.03\). Ventricular volume ratios monotonically increased after stroke for all aged rats (D). However, the increasing rate was slower in the treated group and was significant at 6 weeks after stroke.
Using the sections with Bielschowsky silver and Luxol fast blue-stained sections. Bars in (B) and (D) are 50 μm.

ANOVA demonstrated that the stroke severity was balanced between the groups at the baseline (at day 1 with *P*<0.33). For T2* measurements, no group-by-time interaction was observed (*P*=0.63); however, there was a significant group effect and time effect with *P*<0.01, respectively. Subgroup analysis showed treatment effect on T2* at weeks 4 and 5. The overall treatment effect was observed on CBF at 6 weeks (*P*<0.05), and no treatment-by-time interaction was observed for CBF. As for DA, there was no group-by-time interaction (*P*=0.94); however, overall group effect and time effect were observed, respectively. Subgroup analysis showed a significant treatment effect at weeks 4, 5, and 6, respectively. DA increased as time increased in both groups. The significant difference of group-by-time interaction was detected (*P*<0.01) in volume expansion of ventricle. The sildenafil treatment reduced expansion of the ipsilateral ventricle at 6 weeks after stroke compared to controls (*P*<0.03). As a baseline, we measured the volumes of the contralateral ventricle for all animals with T2 maps, and no significant differences were observed at 6 weeks after stroke between the 2 groups (*P*>0.4).

Histological measurements along the ischemic boundary zone were consistent with MRI results. Using the endothelial barrier antigen-stained sections, the microvascular density of the representative treated aged rat (Figure 4A) was increased compared with a control aged rat (Figure 4B), with the measurements of 462.5±86.9 mm⁻² and 315.3±45.4 mm⁻² for the sildenafil-treated and control groups (*P*<0.05), respectively. Using the sections with Bielschowsky silver and Luxol fast blue staining, the axonal length and density at the same location were longer and higher in the treated aged rat (Figure 4C) than in the control aged rat (Figure 4D), with the measurements of 28.9±3.0% versus 20.6±4.3% of field of view for the sildenafil-treated and control groups (*P*<0.05).

Neurological tests demonstrated that the modified neurological severity score was improved at week 6 in the sildenafil-treated group (Table) compared to controls (*P*<0.04). The modified neurological severity score decreased as time increased (*P*<0.01) in both groups. No significant differences were found for adhesive removal and foot-fault tests between the 2 groups to 6 weeks after stroke (data are not shown).

**Discussion**

This study demonstrated that treatment of embolic stroke with sildenafil in aged rats starting at 24 hours and continuing daily for 7 days significantly promoted angiogenesis detected by hypointensity region on T2* map and axonal remodeling detected by hyperintensity area on DA map, and increased local cerebral blood flow and reduced expansion of the ipsilateral ventricle in the ischemic recovery area at 6 weeks after stroke compared to control aged rats treated with saline. Concomitantly, neurological outcome by modified neurological severity score evaluation was significantly improved after sildenafil treatment of stroke in aged rats.

In a previous study with young adult rats, T2* map provided evidence of ongoing angiogenic cerebral tissue after stroke with hypointensity regions on T2* maps along the ischemic boundary.8 Low-value areas on the T2* map may result from the increase of microvascular density because of angiogenesis and leakage of the newly formed blood vessels because of the incomplete blood–brain barrier. Because the coupling of angiogenesis with neurogenesis by vascular endothelial growth factor and improvement of the microenvironment after angiogenesis, axonal remodeling can be detected using DA map.9 Hyperintensity areas in the DA map along the ischemic boundary after stroke primarily result from increases of axonal density and directionality. The increase of axonal density in rat brain after stroke could be caused by axonal outgrowth and remyelination. Volume expansion of the ipsilateral ventricle after stroke is a sensitive index for assessing the cerebral tissue restoration from stroke damage.12 Damage and loss of brain tissue after stroke may result in the expansion of ventricle. Treatment of stroke that enhances brain remodeling may consolidate the cerebral tissue because of angiogenesis and axonal remodeling and thereby reduce the ventricular expansion and concomitant brain atrophy.

In the current study with aged rats, a low-intensity region on the T2* map in the sildenafil-treated rat was detected at 4 weeks after stroke (Figure 2A) and the increased CBF was present in the same location 1 week later (Figure 2B). The
Spatial and temporal consistencies of the T2* and CBF maps support the hypothesis that the T2* map can detect ongoing angiogenesis in cerebral tissue and the angiogenesis results in an increase of local CBF within the angiogenic region. With the T2* measurements, the differences of angiogenesis in aged rats between the control and treated groups were significant at 4 to 5 weeks after stroke (Figure 3A). Angiogenesis in sildenafil-treated aged rats appear to approach completion at 4 weeks after stroke because the T2* value was minimum at 4 weeks and the regional CBF exhibited a significant difference between the groups 2 weeks later (Figure 3B). Furthermore, a hyperintensity area in the DA map was identified after detection of angiogenesis in the same location (Figure 2C). This suggests a coupling between angiogenesis and axonal remodeling. DA values monotonically increased starting from 1 week after stroke in the aged rats (Figure 3C), which suggests that axonal remodeling starts from 1 week after stroke. The sildenafil treatment significantly enhanced the axonal remodeling in aged rats at 4 weeks after stroke compared to control aged rats.

These brain remodeling events suggest that the functional microvessels after angiogenesis increase local CBF and support the remodeling of neuronal fibers, which may rebuild the microstructure and increase the cerebral tissue density along the ischemic boundary. Thus, the reparative cerebral tissue may resist the ventricular expansion after stroke. In the control aged animals, MRI images did not exhibit apparent angiogenesis, elevation of CBF, and reorganization of neuronal fibers up to 6 weeks after stroke in contrast to sildenafil-treated aged animals. Therefore, the ischemic cerebral tissue in the control rats may be unable to resist the ipsilateral ventricular expansion. Consequently, the reparative cerebral tissue in sildenafil-treated aged rats significantly reduced the expansion rate of the ipsilateral ventricle at 6 weeks after stroke compared with control aged rats (Figure 3D).

Brain remodeling after ischemia may be age-dependent. Compared with young adult rats, neurorestorative processes after stroke may be slower and weaker in aged rats.3 We have previously demonstrated8,9 that hypointensity regions on T2* maps, which characterizes cerebral tissue with ongoing angiogenesis, were present 2 to 3 weeks after stroke in young rats with or without sildenafil treatment, respectively. In the current study of aged rats, the angiogenic tissue was first detected on T2* maps at 4 weeks after stroke in the sildenafil-treated group, which is delayed compared to young animals.9 The elevation of regional CBF and MRI features of axonal remodeling in sildenafil-treated rats were identified on CBF and DA maps, correspondingly, later in the aged rats than in young rats. For most control aged animals, T2* and DA features of angiogenesis and axonal remodeling, however, were not apparent on the T2* and DA maps with the naked eye at least to 6 weeks after stroke.

In our experimental studies, aged rats (18 months) were bigger than young rats (2–3 months). The increased weight of rats may cause smaller ischemic lesion volumes after stroke in aged rats (with 27%±8% of the ipsilateral hemisphere) than in young rats (with 65%±18% infarction) because the same lengths of the embolic clot (4 cm) were used in the middle cerebral artery occlusion stroke model for all young and aged rats. The smaller ischemic lesion volumes in aged rats may partly affect the temporal profile of brain remodeling, causing a weaker restorative response after stroke as compared with young rats. A longer embolic clot in the stroke model in the aged animals, which would produce a larger infarction to be comparable with young rats, would increase the mortality rate (data not shown) to untenable levels.

Conclusions

In summary, the present study demonstrated that sildenafil provides an effective therapy for stroke in the aged rats, although the therapeutic response to the sildenafil treatment was weaker and delayed in the aged rats compared to the young adults. Treatment of embolic stroke in aged rats with sildenafil administered 24 hours and subsequently daily for 7 days after stroke significantly augmented angiogenesis and axonal remodeling, which increased local blood flow and reduced expansion of the ipsilateral ventricle 6 weeks after stroke compared to control aged rats. MRI can be used to investigate and monitor cerebral tissue undergoing angiogenesis and axonal remodeling after stroke in aged rats with or without the sildenafil treatments.

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Disclosures

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