Neuroprotective Effects of Leonurine on Ischemia/Reperfusion-Induced Mitochondrial Dysfunctions in Rat Cerebral Cortex

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Ischemic brain is particularly susceptible to free radicals mediated secondary neuronal damage, especially mitochondrial dysfunctions. Chinese Herbal Medicine with antioxidant properties is believed to have potential therapeutic effect. Leonurine, an alkaloid present in Herba Leonuri (HL), has shown biological effects such as antioxidant, anticoagulant, anti-apoptosis and protection against ischemic heart disease. In this study, neuroprotective effects of leonurine against cerebral ischemia/reperfusion-induced mitochondrial dysfunctions in cortex were evaluated. We used transient rat middle cerebral artery occlusion (MCAO) model of brain ischemia. The rats were treated with their respective treatments for 1 week prior to the MCAO. We found that leonurine significantly improved neurological outcome and reduced ischemia/reperfusion (I/R)-induced cerebral infarction 24 h after MCAO. Leonurine decreased reactive oxygen species (ROS) level in mitochondria isolated from ischemic cortex, which was increased by MCAO. Terminal deoxyuridine triphosphate (dUTP) Nick-End Labeling (TUNEL) staining showed anti-apoptotic effect of leonurine on ischemic cortex. Western blot analysis showed a marked decrease in the expression of Bax and an increase of Bel-2 as a result of leonurine treatment. The attenuation of mitochondrial membrane swelling, restore of mitochondrial membrane potential and content of cytochrome c (Cyt-C) in mitochondria isolated from ischemic cortex could also be observed in leonurine treated group. The findings of this study suggest that leonurine has promising therapeutic effect for ischemic stroke treatment through antioxidant and anti-apoptotic mechanisms.

Key words ischemia; Leonurine; apoptosis; mitochondrial dysfunction

Stroke is the second leading cause of death in industrialized countries and the most important cause of acquired adult disability.1,2 The only current effective available stroke therapy is Thrombolysis but it is limited to only about 5% of stroke patients as it carries the risk of intracranial hemorrhagic transformation.3 Therefore, neuroprotective agents with an extended therapeutic window and the ability to prevent multiple neurochemical cascades which ultimately cause irreversible brain damage are urgently required.

Many studies had clearly shown the involvement of oxidative stress in pathophysiology of cerebral ischemia and stroke.4,5 These studies shown that increased levels of reactive oxygen species (ROS) are the major cause of tissue injury after cerebral ischemia. The elevated ROS level and subsequently the inactivation of antioxidant enzymes and depletion of exiting antioxidants (because of the high level of ROS) resulted in the total breakdown of the endogenous antioxidant defense mechanisms and thus the failure in protecting the neurons from oxidative damage. Recent studies had shown that mitochondrial-formed oxidants are mediators of molecular signaling in the mitochondria-dependent apoptotic pathway, which involves pro- and anti-apoptotic protein binding and the release of cytochrome c (Cyt-C). The activation of this pathway will lead to neuronal death by apoptosis, this suggests that mitochondria may play a vital role in cerebral ischemia.4,6

Herba Leonuri (HL), also named Chinese Motherwort, has been widely used in China as traditional Chinese medicine to treat dysmenorrhea, menoxenia and gynecological disorders in woman.7,8 Our previous study showed that the raw HL extract has neuroprotective effects on middle cerebral artery occlusion (MCAO) rats through its antioxidant and anti-apoptotic effect.9 Recently, raw HL extract was purified and found to consist of mainly Stachydrine (C17H17NO2), Quercetin (C15H10O7), Kaempferol (C15H10O6), Leonurine (C14H21N3O5) and Apigenin (C15H10O5). Leonurine (4-guaiadino-n-butyl-syringate), an alkaloid, was reported to have utoresetnic action and anti-platelet aggregation activities.7,8 We have also reported that Leonurine preserves the antioxidants and does have cardioprotective effect on ischemic myocardium. Leonurine protection on cardiac muscles is thus through its antioxidative and anti-apoptotic effects.8,9

In our present study, the therapeutic effect of Leonurine on ischemic stroke was evaluated. Our studies focused on the antioxidant effect of Leonurine on mitochondria and its anti-apoptotic effect on rats subjected to transient middle cerebral artery occlusion. We employed transient MCAO model of brain ischemia to induce cerebral infarction and performed experiments on rat cortex where many biochemical alterations were supposed to occur.

MATERIALS AND METHODS

Chemical Synthesis of Leonurine Leonurine was synthesized from syringic acid by carbonylation, reaction with thionyl chloride (SOCl2), and the Gabriel reaction, as previously described.10 Leonurine was confirmed to reach 99% purity by high performance liquid chromatography (HPLC). The chemical structure is shown in Fig. 1.

Animal Care All animals were afforded by animal centre of Fudan University and experiments were approved by the Animal Research Ethics Committee, School of Phar-
macy, Fudan University.

**Experimental Protocol** Male Sprague-Dawley (SD) rats weighing 180—220 g were housed under diurnal lighting condition and allowed food and water *ad libitum*. All the animals were randomly divided into 3 groups: sham-operation (Sham); MCAO group with water treatment (Vehicle); MCAO group treated with 60 mg/kg/d of Leonurine (Leo). The drugs were administered orally once daily before MCAO surgery. After 1 week of presurgery treatment, stroke was induced in the rats by MCAO ischemia/reperfusion as previously described. Rats were sacrificed 24 h after surgery for the isolation of mitochondria from cortex. Mitochondria swelling, mitochondria membrane potential and Cyt-C release were estimated in isolated mitochondria in various groups. In addition, terminal deoxynucleotidyl triphosphatase (dUTP) Nick-End Labeling (TUNEL) staining and expression of Bax and Bcl-2 proteins were also assayed.

**In Vivo Ischemic Stroke Model** Male adult SD Rats were anaesthetized with ketamine/xylazine mixture (0.1 ml/100 g intraperitoneally (i.p.)). The left MCAO was induced with the reported method. In brief, the left common carotid artery was exposed through a midline incision in the neck. The external carotid artery was then tied closed. A 3-0 suture with blunt end was introduced into the common carotid artery and advanced into the middle cerebral artery via the internal carotid artery (ICA) (20—22 mm) until a slight resistance was felt. At this point, the blood flow of the middle cerebral artery was occluded. Two hours after the induction of ischemia, the suture was slowly withdrawn and the animals were then returned to their cages and maintained until needed. The neurobehavioral deficits were elevated and brains of each rat were harvested at 24 h after MCAO.

**Measurement of Infarct Volume** The infarct volume was assessed with TTC (2,3,5-triphenyltetrazolium chloride) staining. After the brains were collected, cerebellum and overlying membranes were removed. The brains were then sectioned into eight pieces of 3 mm thick coronal slices using a brain-sectioning block. The coronal sections were stained with 0.1% TTC solution at 37 °C for 30 min and preserved in 4% formalin solution. The infarct size was analyzed with an image analyzer system (Scion image for windows) and converted by integration (including correction for edema and atrophy) to the true infarct size of ischemic damage in the whole hemisphere as previously described.

**Evaluation of Neurological Deficit** To evaluate neurological deficit grading system was carried out as we described previously. A scale of zero to five was used to assess the behavioral and motor changes observed in the animal after the MCAO procedure. Rats were scored zero when they were suspended from the tail, and extended both forelimbs toward the floor, this is an exhibition of normal behavior. For those rats with contralateral forelimb on the side of the MCAO consistently flexed during the suspension and there was no other abnormality observed, they will be assigned a score of one. The rats were then placed on the ground and allowed to move freely and were observed for circling behavior. Rats that moved spontaneously in all directions but showed a monodirectional circling towards the paretic side when given a light jerk of the tail were assigned a score of two. A score of three was assigned to rats that showed a consistent spontaneous contralateral circling. Rats that were very weak and walked only when stimulated were scored as four. If the rats died at the day of assessment, they were assigned a score of five. Rats that showed a higher clinical score exhibited all features of the lower grades, thus were the one that had more severe neuronal function impairment.

**Measurement of ROS Level in Mitochondria** Formation of ROS was evaluated using 2',7'-dichlorofluorescin diacetate (DCFH-DA, Sigma, U.S.A.), a membrane-permeable probe. The non-fluorescent dye freely penetrates mitochondria, was then hydrolyzed by esterases to 2',7'-dichlorodihydrofluorescein (DCFH), and trapped inside mitochondria. Once oxidised by ROS, DCFH yields the highly fluorescent product dichlorofluorescein (DCF). Mitochondria containing 40 μg protein were incubated with 200 μl DCFH-DA buffer (250 mM sucrose, 20 mM Mops, 10 mM Tris, 50 μM ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5 mM Mg2+, 0.1 mM P1(K+), 1.0 mM cyclosporin A, 10 mM DCFH-DA, pH 7.0). One hour after malate and glutamate were added, fluorescence was read at the excitation wavelength of 488 nm and the emission wavelength of 525 nm.

**Measurement of Mitochondrial Swelling** Measurement of mitochondrial swelling was done by the method of Halestrap. Two-hundred microliters of isotonic buffer (5 mM KH₂PO₄, 250 mM sucrose, 3 mM succinic acid di- sodium salt, pH 7.2) was added to mitochondria (50 μg protein) at 30 °C for 5 min. The absorbance was measured at 520 nm.

**Measurement of Mitochondrial Transmembrane Potential (ΔΨm)** Rhodamin 123 is highly specific fluorescent dyes for mitochondria, the intake of Rhodamin 123 depends on the mitochondrial membrane potential. Ten micrograms mitochondria protein suspension was added to 2.5 ml of reaction buffer (15 mM sucrose, 5 mM magnesium chloride, 5 mM sodium succinate, 2.5 mM otenone, 20 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonylic acid (HEPES), pH 7.4), 40 μl of Rhodamin 123 at 26 μmol/l was then added and incubate for 5 min at room temperature. Mitochondria were then pelleted by centrifugation and the fluorescence of the supernatant was read with a spectrophotometer at the excitation wavelength at 503 nm and the emission wavelength of 527 nm.

**Cyt-C in Mitochondria** Cyt-C was measured by the method of Zhang. Briefly, 10 μl mitochondrial protein and
190 μl phosphate buffer were added in to 96 wells plate. Then, sodium hyposulfite was added. The absorbance at 520 nm was measured with a single wave spectrophotometer and the content of Cyt-C in mitochondria can be obtained according to the Cyt-C standard curve.

**Western Blot Analysis for Bax and Bcl-2** Cortices were harvested, weighed and homogenized (10%) in ice cold RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China). Soluble proteins were collected by centrifugation at 12000 g. Protein lysates were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corp.). After blocking with 5% skim milk, the membranes were incubated with the respective primary antibodies (Bcl-2, 1:200; Bax, 1:200, Santa Cruz, U.S.A.) in phosphate buffered saline (PBS) 0.1% Tween-20 overnight at 4 °C. The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) antibodies at a 1: 10000 dilution (Jackson ImmunoResearch Laboratories Inc., U.S.A.). Immunoreactive proteins were then visualized using enhanced chemiluminescence (ECL). The signals were quantified by densitometry using a Western blotting detection system (Alpha Innotech, U.S.A.) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the loading control.

**Terminal dUTP Nick-End Labeling Assay** At the end of treatment, the brains were collected and post-fixed in 2% paraformaldehyde for 2 h. They were then transferred into 30% sucrose in phosphate buffer to allow the sample to dehydrate. Cortex tissues were cut at 20 μm using a Leica CM1510 Cryostat (Leica Microsystems, IL, U.S.A.). The TUNEL assay was conducted by using the DNA Fragmentation Detection Kit (Promega, U.S.A.) according to the manufacturer’s instruction. In brief, the slides were incubated with 0.05% proteinase K for 5 min and endogenous peroxidases were inactivated by 0.3% hydrogen peroxide in methyl alcohol for 10 min. Slides were then incubated with equilibration buffer followed by terminal deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at 37 °C for 1 h, and a stop/wash buffer was applied for 30 min at 37 °C. The slides were incubated with digoxigenin peroxidase-conjugated antibody, which can be visualized with diaminobenzidine (DAB) substrate. The sections were counterstained with hematoxylin. In the TUNEL stained slides, 3 fields for each section were selected from cerebral cortex. TUNEL-positive cells were quantified by light microscopy at magnification (×200). The total cell numbers and TUNEL-positive cell numbers were obtained in each field. The percentage of TUNEL-positive cells is described as the percentage of the numbers of TUNEL-positive cells to the total numbers of cells in each field.

**Protein Estimation** Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China).

**Statistical Analysis of Data** Data were represented as mean±S.E.M. of at least three independent preparations. Statistical analysis was performed by the one way analysis of variance (ANOVA) with Tukey’s post hoc test. A difference with p<0.05 was considered statistically significant.

**RESULTS**

**Infarct Volume and Neurological Deficit** Infarct volume of each treatment group is shown in Figs. 2A and B. As expected, the rats from sham had no cerebral injury, therefore no infarct area was observed. When the animal was subjected to ischemia insult by MCAO, the infarct area was observed in cortex and striatum. With the treatment of Leonurine, the infarct volume was reduced significantly from 25.45±2.21% to 16.19±1.28% (Fig. 2B). When evaluating neurological function, neurological deficit grading system was carried out for all the animals. The higher the neurological deficit score, the more severe impairment of motor motion. The result is shown in Fig. 2C. Rats from sham group did not have any neurological deficit, and therefore throughout the entire study, the animals had the neurological score of zero. For the rats in vehicle group, they remained highest neurological deficit score after the surgery. This is in agreement with the fact they had the largest infarct volumes among the three groups (Figs. 2B, C). With the pretreatment of Leonurine, neurological deficit score of rats after MCAO was lowered as compared to vehicle group (2.00 vs. 2.88) at day 1 (Fig. 2C).

**ROS Generation in Mitochondria** ROS level in mitochondria is expressed as the percent of sham group, which is shown in Fig. 3. Compared with sham group, vehicle group showed increased ROS production in mitochondria. In contrast, pretreatment with Leonurine significantly reduced the increase in ROS generation (Fig. 3).

**Mitochondrial Membrane Swelling** Measurements of mitochondrial membrane swelling in three groups are shown in Fig. 4. Membrane swelling was measured at 24 h after surgery. Six rats from each group were randomly selected for the measurement of mitochondrial membrane swelling. It was observed that mitochondria from vehicle group (0.30±0.055) showed a significant decrease compared with sham group (0.71±0.072) in absorbance which can be restored by Leonurine pretreatment (0.59±0.045).

**Mitochondrial Membrane Potential** Isolated Mitochondria were incubated with Rhodamine 123 for 5 min before they were pelleted and the supernatant of the incubation was collected for fluorescence spectrophotometry. The fluorescence intensity of the supernatant is inversely proportional to the mitochondrial membrane potential (Fig. 5). When membrane potential is high, mitochondria will retain the Rhodamin 123 dye more and the fluorescence intensity of the supernatant will be lower. If there is a loss of membrane potential, mitochondrial membrane can no longer hold on to Rhodamine 123 and the dye is free to leak out of mitochondria and the fluorescence intensity in the supernatant will be higher. In the vehicle group, the fluorescence intensity in the supernatant was greatly increased compared with sham group, which can be significantly decreased by Leonurine pretreatment. This indicated a loss of mitochondrial membrane potential during MCAO induced stroke and the restoration of that membrane potential by Leonurine.

**Cyt-C Release** Figure 6 shows that concentration of mitochondrial Cyt-C in vehicle group were decreased from 95.23 μg/mg protein to 49.76 μg/mg protein as compared with sham group. Leonurine treatment can reverse the mitochondrial Cyt-C from 49.76 μg/mg protein to 80.53 μg/mg protein (Fig. 6).
Fig. 2. Neuroprotective Effect of Leonurine on Cerebral Ischemia in Rats

(A) Leonurine reduces cortical and subcortical infarct size in the rats stroke model. Serial coronal brain sections with TTC staining at 24 h after MCAO. (B) Quantitative analysis of infarct volume. **p<0.01, compared with sham group; #p<0.01, compared with vehicle group, n=8. (C) Leonurine decreased the score of neurological outcome at 24 h after MCAO. **p<0.01 compared with sham group; #p<0.05 compared with vehicle group, n=12.

Fig. 3. Effect of Leonurine on Cerebral Ischemia/Reperfusion-Induced ROS Generation of Mitochondria

Leonurine can significantly decrease mitochondrial ROS generation induced by cerebral ischemia/reperfusion. **p<0.01 compared with sham group. **p<0.01 compared with vehicle group.

Fig. 4. Effect of Leonurine on the Mitochondrial Membrane Swelling of Left Cortex in MCAO Rats

Mitochondria from vehicle group showed a decrease in absorbance when compared with sham group, absorbance can be elevated by Leonurine treatment. **p<0.01 compared with sham group, #p<0.05 compared with vehicle group, n=6.
Expression of Bcl-2 and Bax Proteins Tested by Western Blot

Expression levels of apoptosis-related proteins were examined by Western Blot. Bcl-2 protein is known to promote cell survival as well as to suppress cell death by various apoptotic stimuli. Bax, a proapoptotic protein, can integrate into the outer mitochondrial membrane in response to apoptotic stimuli inducing Cyt-C release via mitochondrial permeability transition pore (MPTP) formation. In this study, MCAO induced apoptosis was accompanied by a decrease in the expression of Bcl-2 (0.56\(\pm\)0.09) and an increase in the Bax expression (0.95\(\pm\)0.13). (Fig. 7). The difference in Bax expression was statistically significant (\(p<0.05\)). Pre-treatment with 60 mg/kg Leonurine prevented the decrease in the expression of Bcl-2 protein whereas it suppressed the increase of Bax expression. Our results indicated that Leonurine had anti-apoptotic effect. The protein band from each group was shown in Fig. 7A. In the picture, we can see Bax expression is significantly up-regulated and Bcl-2 is significantly down-regulated with MCAO, but both proteins expressions could be reversed by Leonurine pretreatment.

Terminal dUTP Nick-End Labeling Assay

The apoptotic cells were identified through TUNEL staining. Leonurine significantly reduced apoptotic cell death caused by MCAO. The number of TUNEL positive cells was significantly increased in the ischemic damage region of vehicle-treated animals, which was reduced in the cerebral cortex of Leonurine-treated group (Figs. 8A—C). The proportion of TUNEL positive cells was 65.3\(\pm\)6.5\% and 27.7\(\pm\)4.4\% in the cerebral cortex of vehicle- and Leonurine-treated animals, respectively (Fig. 8D).

DISCUSSION

Our data presented neurochemical and neurobehavioral ev-
idences that Leonurine could protect against cerebral I/R-induced secondary neuronal damage by preserving mitochondrial function in cortex. Pretreatment of Leonurine at 60 mg/kg/d could significantly reduce the infarct volume and alleviate the neurological impairment, indicating the lesser histological damage compared to the vehicle group. This prompted us to further our study to evaluate the possible therapeutic mechanisms of Leonurine. Mitochondria are essential organelles involved with oxidative phosphorylation, calcium homeostasis, reactive oxygen species (ROS) management, and apoptosis. Convergence of a number of cell death pathways emanating from membrane receptors, the cytosol, nucleus, lysosome, and endoplasmic reticulum on the mitochondria results in mitochondrial destabilization. A common consequence of these death pathways is damage to the mitochondria. Given the prominent role of mitochondria in triggering apoptosis pathways, we hypothesized that identifying drugs that target mitochondrial pathways may be broadly neuroprotective.

Oxidative stress had been extensively studied in relation to pathophysiology of cerebral ischemia, and reactive oxygen species (ROS) had been found to be overproduced during ischemia/reperfusion (I/R) of neural tissues. In ischemic brain, the excessive production of ROS can cause cellular damage and subsequent cell death. Mitochondria are the primary cellular consumer of oxygen and contain multiple electron carriers and redox enzymes that can transfer the single electrons to oxygen, generating O₂⁻. Therefore, mitochondria are considered as the primary intracellular sources of ROS. Not only producing ROS, mitochondria are also the targets of oxidative stress. Mitochondria energy metabolism is extremely sensitive to impairment by free radicals and that mitochondrial oxidative stress limits metabolic recovery. Dysfunctional mitochondria will produce more ROS, and a feed-forward loop is set up whereby ROS-mediated oxidative damage to mitochondria favors more ROS generation, resulting in a vicious cycle. In addition to the energy state and ion homeostasis, mitochondrial function is disrupted by ROS during cerebral ischemia. Cerebral I/R-induced ROS mediated oxidative damage contributes in the exacerbation of intracellular calcium levels leading to mitochondrial swelling that subsequently leads to the opening of the mitochondrial permeability transition pores and depolarization of mitochondrial membrane, which results in the release of mitochondrial components (i.e., cytochrome c and apoptosis-inducing factor), which in turn initiate apoptosis. Therefore, modulation of mitochondrial function by antioxidant would be ideal therapy for ischemic stroke.

As a result of Leonurine treatment, a significant decrease in mitochondrial ROS was observed, which could be attributed to the antioxidant potential of Leonurine. The reduced mitochondrial ROS may attenuate apoptosis through modulating mitochondrial function. Present study identified apoptotic cells with TUNEL staining. Leonurine treatment significantly decreased the number of TUNEL positive cells after cerebral ischemia. Furthermore, this study suggested that Leonurine exerted its anti-apoptotic effect by preserving mitochondrial function, which could be related to its antioxidant potential.

Mitochondrial transmembrane potential, which is the potential difference caused by the difference in the ion concentration on the two sides of the mitochonaria membrane, is a sensitive indicator reflecting the mitochondrial function. The decline of mitochondrial transmembrane potential was temporally correlated with the opening of the permeability transition pore, leading to the release of caspase-activating proteins. Long-lived pores can also cause swelling of mitochondria and, as a consequence, the disruption of the outer mitochondrial membrane. Therefore, mitochondrial swelling can also reflect the opening of the permeability transition pore. In this study, MCAO group showed a significant increase in mitochondrial swelling and a significant decrease in mitochondrial membrane potential. Interestingly, Leonurine treatment could maintain the mitochondrial membrane potential and decrease mitochondrial swelling similar to sham-operated group, which suggested the inhibition of mitochondrial membrane permeability.

Interactions between the proapoptotic and antiapoptotic Bcl-2 family proteins on the outer mitochondrial membrane are believed to play an important role in cell survival. Bax has an extensive amino acid homology with Bcl-2. Bax homodimerizes and forms heterodimers with Bcl-2. Cell fractionation and confocal microscopy showed that Bax localized in the cytosol of most cells, although it has the C-terminal putative transmembrane domain, similar to that of Bcl-2. With apoptotic stimuli, Bax is post-transcriptionally activated, then it oligomerizes and translocates to mitochondria. Mitochondrial Bax triggers Cyt-C release from mitochondria. Cyt-C is a post mitochondrial activator of apoptosis, downstream to the pre-mitochondrial mechanisms. As such, mitochondrial Cyt-C is released from the intermembrane space, activating cytosolic reaction cascades encompassing caspases and regulatory factors, which eventually will lead to cytoskeletal degradation and DNA fragmentation. Cyt-C release activates downstream caspases of the intrinsic pathway through formation of the apoptosome, a complex of dATP, cytochrome, procaspase 9 and Apaf1. Our studies show a significant decrease in mitochondrial concentration of Cyt-C in MCAO group, which may be due to the up regulated Bax level and down regulated Bcl-2 level. This decrease in mitochondrial Cyt-C level was markedly altered in Leonurine treated group. This could be summarized that Leonurine effectively scavenged mitochondrial ROS generation in ischemic cortex and hence maintained mitochondrial functions, which subsequently attenuated apoptosis through inhibition of Bax-induced Cyt-C release from mitochondria.

To the best of our knowledge, this is the first demonstration of the therapeutic potential of Leonurine on brain injury induced by ischemia/reperfusion. Although the underlying mechanisms and the feasibility of long term usage are yet to be verified, the data suggested that Leonurine was an excellent antioxidant, which exerted its neuroprotective effect against cerebral ischemic injury through preserving mitochondrial function, therefore, a therapeutic potential for treatment of stroke.

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