Delayed administration of guanosine improves long-term functional recovery and enhances neurogenesis and angiogenesis in a mouse model of photothrombotic stroke

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Abstract. Guanosine (GUO) is neuroprotective when administered acutely for the treatment of cerebral ischemia. The aim of the present study was to investigate whether delayed administration of GUO improved long-term functional recovery following stroke, as well as to explore the potential underlying mechanisms. GUO (8 mg/kg) or a vehicle was administered intraperitoneally for 7 consecutive days beginning 24 h prior to photothrombosis-induced stroke in male C57/B6J mice. Behaviour tests were performed at days 1, 3, 7, 14 and 28 post-stroke. Infarct volume was measured using Nissl staining at day 7 post-stroke. Neurogenesis and angiogenesis were evaluated by co-labelling bromodeoxyuridine (BrdU) with doublecortin (DCX), neuronal nuclei (NeuN) and von Willebrand factor, in immunohistochemical studies. Brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) levels in the ipsilesional brain at day 28 post-stroke were detected by western blot analysis. Delayed administration of GUO did not reduce infarct volume or affect neurological function at day 7 post-stroke; however, it did improve functional recovery from day 14 post-stroke, when compared with the vehicle group. GUO significantly increased the number of $BrdU^+$ and $BrdU^+/DCX^+$ cells in the subventricular zone and subgranular zone at all examined time points, the number of $Brdu^+/NeuN^+$ cells in the peri-infarction region at days 14 and 28 post-stroke and microvessel density in the peri-infarction region at day 28 post-stroke compared with the vehicle group. In addition, the BDNF and VEGF levels in the ipsilesional brain were significantly elevated. Delayed administration of GUO at 24 h post-stroke enhanced neurogenesis and angiogenesis, and increased BDNF and VEGF levels, which likely contributes to long-term functional recovery following stroke.

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Introduction

Stroke is a major cause of death and disability worldwide, however treatment options remain limited (1,2). A small fraction of patients benefit from administration of recombinant tissue-type plasminogen activator; however, this has a short time window, as well as additional limitations (3) In a previous study, brain remodelling and plasticity, including neurogenesis and angiogenesis, has emerged as a novel promising therapeutic target for stroke (4). Persistent neurogenesis in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus may be stimulated by cerebral ischemia and other injuries as part of the endogenous repair response (5). Angiogenesis, the formation of new microvessels from the existing vasculature, improves tissue microperfusion in the peri-infarction region following a stroke (6).

Guanosine (GUO), a guanine-based purine, serves several important roles in the central nervous system (7,8). Endogenous GUO levels increase within 2 h of focal stroke and remain elevated for 7 days (9). An accumulating body of evidence indicates that exogenous administration of GUO prior to or immediately following experimental stroke confers acute neuroprotection following cerebral ischemia (10-14). However, any benefits to an ischemic stroke from delayed treatment of GUO remain unknown. Notably, GUO has been reported to promote neurite outgrowth from PC12 cell cultures (15) and proliferation of neural stem cells (16). In addition, GUO has been indicated to promote myelination in a murine model of spinal cord injury (17) and induce synaptogenesis in the brain of healthy animals (18). These results suggest that GUO may serve as a restorative target.

Therefore, the present study involved investigation of whether delayed treatment with GUO (24 h following stroke) improves the long-term functional outcome following a stroke, as well as exploring the possible mechanisms underlying GUO restorative effects.

Materials and methods

Animals and experimental model of photothrombotic stroke. The experiments were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), and were in accordance with the guidelines of the Institute of Laboratory Animal Resources (Washington, DC, USA). A total of 120 male C57BL/6J wild-type mice (weight, 20-25 g; age, 8-10 weeks old) were purchased from the Tongji Medical College Experimental Animal Centre (Wuhan, China). Animals were housed in a temperature- and humidity-controlled environment with a 12 h light/dark cycle and free access to food and H_2O. Focal stroke was induced by photothrombosis (PT) as described previously (19). Mice were anesthetized using 10% chloral hydrate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) (35 mg/kg) intraperitoneally and placed in a stereotactic apparatus. A midline incision of the skin exposed the skull. A dose of 0.1 ml rose bengal solution (10 mg/ml in normal saline; Sigma-Aldrich; Merck KGaA) was injected intraperitoneally 5 min prior to illumination. For illumination, a cold light source (KL1500 LCD; Zeiss AG, Oberkochen, Germany) with a 4-mm aperture was centred 2 mm lateral from the bregma. The brain was illuminated through the intact skull for 15 min. All mice survived the procedure and exhibited behavioural deficits.

Drug administration. All animals subjected to PT-induced stroke were randomly assigned to receive GUO or vehicle following stroke induction. Researchers were blinded to experimental groups. GUO (0.5 mg/ml in sterile saline; Sigma-Aldrich; Merck KGaA) was administered intraperitoneally (i.p.; 8 mg/kg) beginning 24 h following the stroke and then daily for 7 days. The vehicle group received an equal volume of saline. Bromo-deoxyuridine (BrdU; 10 mg/ml in sterile saline; Sigma-Aldrich; Merck KGaA) was injected i.p. (100 mg/kg) beginning 24 h following the stroke and then twice daily until the animals were sacrificed.

Measurement of infarct volume. The animals were sacrificed 7 days post-stroke. Anesthetized mice were transcardially perfused with cold PBS followed by 4% paraformaldehyde. Brains were subsequently removed, fixed in fresh 4% formaldehyde solution at 4˚C overnight and immersed in 30% sucrose until they sank. Then the brains were frozen at -80˚C. The frozen brains were cut into 10-μm coronal sections on a cryostat (CM3050S; Leica Microsystems GmbH, Wetzlar, Germany). For each brain, 15 sequential sections were taken at 100-μm intervals and processed for Nissl staining. Sections were stained with 0.1% crystal violet solution for 10 min at room temperature. Infarct volumes were measured using an image analysis program (ImageJ version 1.46r; National Institutes of Health, Bethesda, MD, USA).

Behavioural testing. Behavioural tests were carried out prior to the PT procedure and 1, 7, 14 and 28 days following PT using the modified neurological severity scale (mNSS), grid walking test and cylinder test.

mNSS. mNSS is a composite of motor, sensory, balance and reflex tests. Neurological function is graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). A total of 1 score point is awarded for the inability to perform the test or for the lack of a tested reflex; therefore, a more severe injury has an increased score (20).

Grid walking test. Animals were placed on an elevated wire grid and video-recorded when they walked. The number of contra- and ipsilateral faults for each limb and the total number of steps taken were counted, the ratio between foot faults and total steps was calculated (21).

Cylinder test. The animal was placed in a transparent cylinder and video-recorded. Forelimb preference during vertical exploration of the cylinder was evaluated by recording the forelimb contacts. Animals were subjected to one trial on day 1 prior to PT. The asymmetry score for each animal was calculated by the formula previously described (22).

Immunohistochemistry. Frozen sections were incubated with a blocking buffer (1X PBS/5% normal goat serum/0.3% Triton X-100, Goodbio, Wuhan, China) for 1 h at room temperature. The sections were subsequently incubated at 4˚C overnight with the following primary antibodies: mouse monoclonal anti-BrdU (catalogue no. sc-32323, dilution, 1:100, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit polyclonal anti-BrdU (catalogue no. ab19944, dilution, 1:500, Abcam, Cambridge, MA, USA), goat polyclonal anti-doublecortin (DCX; catalogue no. sc-8066, dilution, 1:200; Santa Cruz Biotechnology, Inc.), mouse monoclonal neuronal nuclei (NeuN; Catalogue no. MAB377, dilution, 1:500; EMD Millipore, Billerica, MA, USA) and von Willebrand factor (vWF; catalogue no. ab7356, dilution, 1:1,000; EMD Millipore). Following washing with PBS, the sections were incubated for 1 h at room temperature with two secondary antibodies: Alexa Fluor 488 (catalogue no. CA11001s, dilution, 1:200; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Alexa Fluor 594 (catalogue no. CA11012s, dilution, 1:200; Invitrogen; Thermo Fisher Scientific, Inc.). Incubation was conducted in the dark and then washed with PBS three times. For BrdU immunofluorescence, brain sections were pre-treated with 2 M HCl at 37˚C for 30 min and then washed with PBS six times at room temperature before being incubated with blocking solution. The images were captured using a fluorescence microscope (BX51; Olympus Corporation, Tokyo, Japan).

Western blot. Mice were anesthetized and decapitated at 14 days post-stroke. The ipsilateral peri-infarct cortices and cognate regions from the contralateral hemisphere were sampled. The total protein of each tissue was extracted according to the instructions of the protein reagent kit (Goodbio, Wuhan, China). Protein concentration of each sample was determined using the bicinchoninic acid assay (Sigma-Aldrich; EMD Millipore). Protein samples (20 μg per lane) were subsequently separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Two primary antibodies were incubated with the membranes overnight at 4˚C: anti-brain-derived neurotrophic factor (BDNF; catalogue no. AB1779, dilution, 1:200; EMD Millipore), anti-vascular endothelial growth factor (VEGF; catalogue no. sc-152, dilution, 1:200; Santa

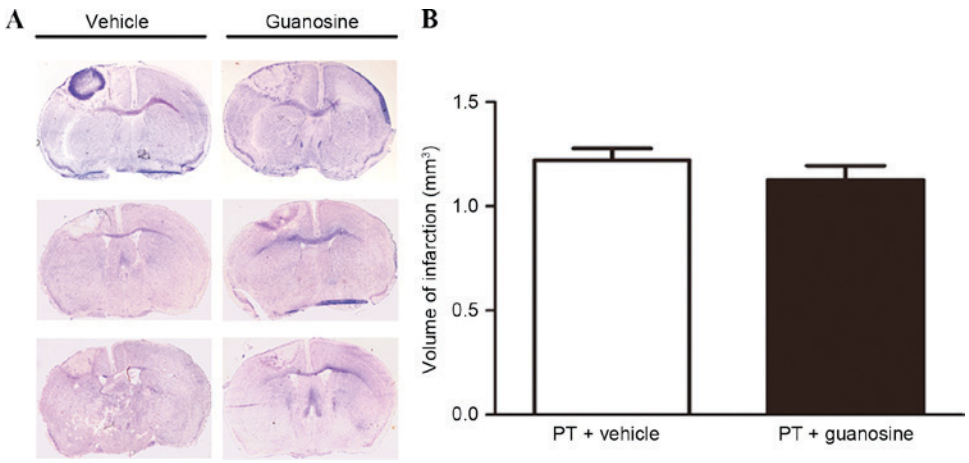

Figure 1. Delayed administration of guanosine does not reduce the infarct volume at day 7 following PT-induced stroke. (A) Representative Nissl stained sections from vehicle- and guanosine-treated mice following 7 days of stroke. (B) Quantification of the infarct volume of vehicle and guanosine group. Data are presented as the mean ± standard error of the mean, n=4 per group. P>0.05 vs. PT + vehicle. PT, photothrombosis.

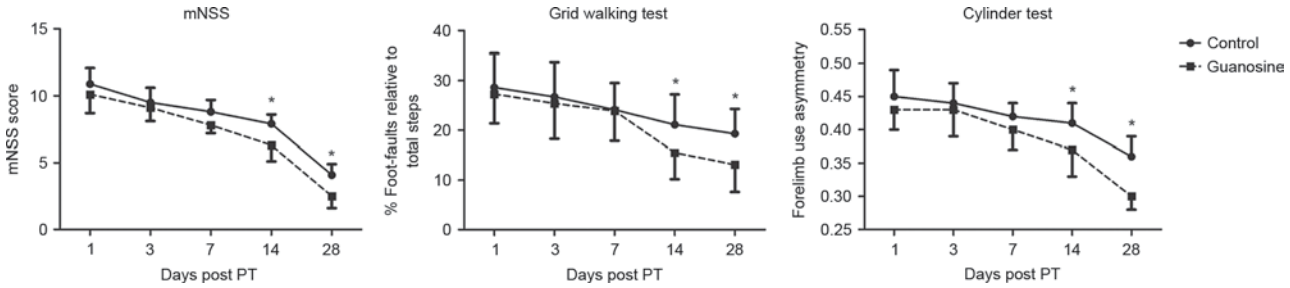

Figure 2. Delayed administration of guanosine improves long-term functional recovery following PT-induced stroke. No significant difference was detected on days 1 and 7 post-stroke in (A) mNSS score, (B) foot fault or (C) forelimb asymmetry between the vehicle group and guanosine group. However, a significant improvement in the mNSS score, foot fault and forelimb asymmetry was observed on days 14 and 28 post-stroke following guanosine treatment. Data are presented as the mean ± standard error of the mean, n=8 per group. *P<0.05 vs. Control. PT, photothrombosis; mNSS, modified neurological severity scale.

Cruz Biotechnology, Inc.) and β-actin (catalogue no. 4970, dilution, 1:1,000; Cell Signalling Technology, Danvers, MA, USA). Subsequently, membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (catalogue no. AB501, dilution, 1:1,000; Novoprotein, Shanghai, China) for 2 h at room temperature. was used as a loading control for all experiments. Densitometry analysis was performed using the Image J software with normalization to β-actin.

Statistical analysis. All data are represented as mean ± standard error of mean and were analysed using SPSS software (version 21.0; IBM SPSS, Armonk, NY, USA). The treatment effects on the behaviour score at different time points were analysed using repeated measures one-way analysis of variance, followed by Tukey-Kramer post hoc tests. When comparing two groups, statistical analysis of data was performed using Student's *t*-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Delayed administration of GUO does not reduce infarct volume but improved the post-stroke long-term functional outcome. There was no statistical difference in infarct volume on day 7 post-stroke between the two groups (1.220±0.110 vs. 1.125±0.120 mm^3, n=8 for each group, P>0.05; Fig. 1). In addition, no difference was detected on days 1 and 7 post-stroke, in mNSS score, foot fault or forelimb asymmetry between the vehicle group and GUO group (Fig. 2A-C). However, a significant reduction in the mNSS score and foot fault was observed on days 14 and 28 post-stroke following GUO treatment (P<0.05; Fig. 2A and B). Similarly, treatment with GUO significantly improved the function of the impaired forelimb on day 14, and the effect was maintained up to 28 days post-stroke. The results indicated that delayed administration of GUO was able to promote long-term functional recovery following ischemic stroke (Fig. 2C).

Delayed administration of GUO enhances neurogenesis in the ischemic brain. GUO significantly increased the number of $BrdU^+$ cells in the ipsilateral SVZ and SGZ when compared with the vehicle group (P<0.05; Fig. 3A-C). These data indicated that administration of GUO enhances cell proliferation following stroke.

In addition, GUO significantly increased the number of $BrdU^+$ cells co-labelled with DCX, a marker of neural progenitor cells, in the ipsilateral SVZ and SGZ compared with the vehicle group (P<0.05; Fig. 3D and E) at all time points following stroke induction, suggesting that delayed administration of GUO promotes proliferation of neural progenitor cells in the SVZ and SGZ following stroke.

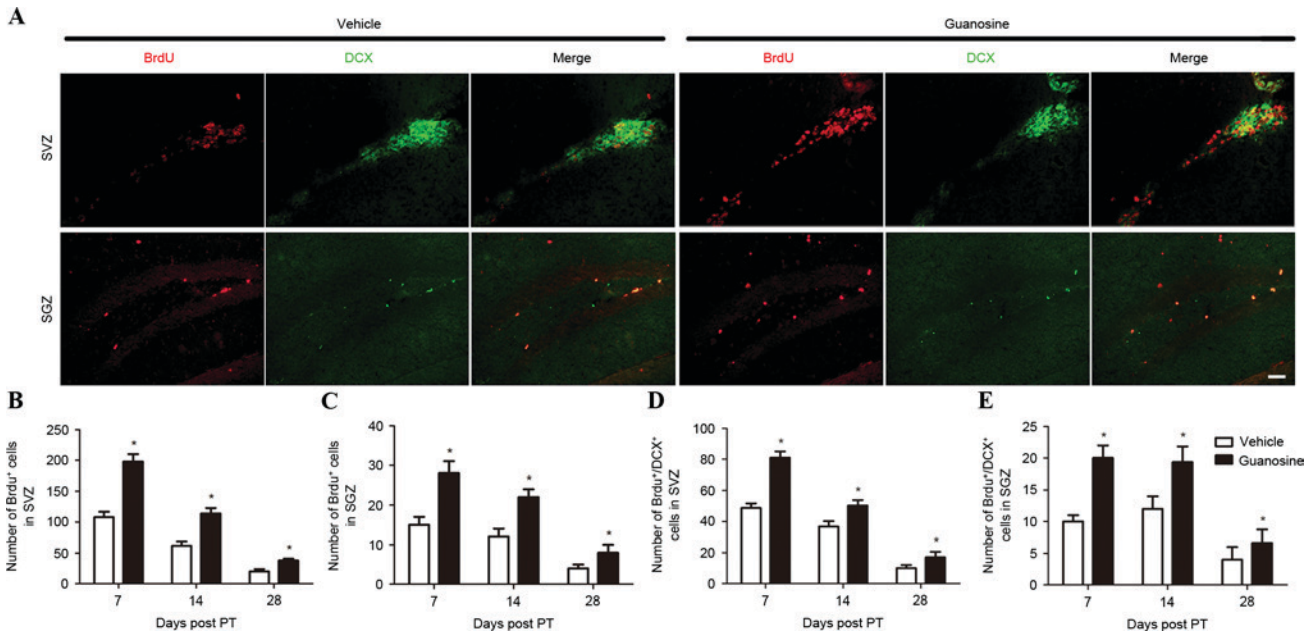

Figure 3. Delayed administration of guanosine promotes neural progenitor cells proliferation in the SVZ and SGZ. (A) Immunostaining of (red) BrdU and (green) DCX in the SVZ and SGZ of vehicle- and guanosine-treated mice at day 7 post-stroke. Quantification of $BrdU^+$ cells in the (B) SVZ and (C) SGZ, and $BrdU^+/DCX^+$ cells in the (D) SVZ and (E) SGZ, at each time point for each group. Data are presented as the mean ± standard error of the mean, n=6 per group. *P<0.05 vs. Vehicle. Scale, 100 μm. SVZ, subventricular zone; SGZ, subgranular zone; BrdU, bromodeoxyuridine; DCX, doublecortin.

To further investigate whether the proliferative neural progenitor cells is able to differentiate into functional neurons, double immunostaining was performed with NeuN (a marker of mature neurons) and BrdU. GUO significantly increased the number of $BrdU^+/NeuN^+$ cells in the peri-infarction cortex following stroke, when compared with the vehicle group (P<0.05; Fig. 4A and B). These data indicated that GUO enhances the differentiation of new neural progenitor cells into mature neurons within the peri-infarction region following stroke.

Delayed administration of GUO enhances angiogenesis in the ischemic brain. To examine whether GUO influences the formation of new blood vessels in the ischemic brain, all blood vessels in the peri-infarction cortex were counted using vWF immunostaining. vWF is a vascular endothelial cell marker. GUO significantly increased vascular density in the peri-infarction cortex compared with the vehicle group (P<0.05; Fig. 4C and D). Furthermore, these results presented a significant increase in the percentage of $BrdU^+/vWF^+$ cells in mice treated with GUO, when compared with the vehicle group (P<0.05; Fig. 4E). These data suggested that GUO enhances angiogenesis following stroke.

Delayed administration of GUO promotes the expression of neurotrophic factors. Western blot analysis demonstrated that GUO significantly increased the expression of VEGF and BDNF in the ischemic brain at day 14 post-stroke compared to the vehicle (P<0.05; Fig. 5).

Discussion

The primary result of the present study was that delayed administration of GUO improved long-term functional outcome following a PT-induced stroke; however, did not reduce infarct volume. In addition, GUO enhanced post-ischemic neurogenesis and angiogenesis, which likely contributed to the restorative effects of GUO. Furthermore, GUO increased the expression of two key neurotrophins, BDNF and VEGF, suggesting that neurotrophic effects may contribute to the enhancing effects of GUO on post-ischemic neurogenesis and angiogenesis.

Treatment with GUO prior to or immediately following experimental cerebral ischemia confers acute neuroprotection in multiple *in vitro* and *in vivo* stroke models (10-14). The mechanisms responsible for the neuroprotective effects may be associated with the anti-oxidative stress, anti-excitatory toxicity and anti-apoptosis activities of GUO (9,10,13,23). In the present study, delayed administration of GUO was investigated, to identify whether it improved long-term functional outcome following a stroke. The results indicated that GUO administered 24 h following PT accelerated long-term recovery. In particular, delayed GUO treatment only improved neurological functions from 14 days following the stroke and did not improve functions during the acute phase, which suggested that delayed GUO treatment may promote functional recovery through restorative rather than acute neuroprotective mechanisms.

In addition, the infarct volume at 7 days following stroke was not reduced. This result is consistent with previous studies in which infarct volume was only reduced by GUO when it was administered within a tight administration schedule (11,12). These results suggest that delayed treatment with GUO did not exert an acute neuroprotective effect on cerebral ischemia, resulting in an unchanged infarct size.

GUO has been indicated to induce neurogenesis in SVZ in a mouse Parkinsonism model (24) and synaptogenesis in the healthy rat brain (18). However, whether GUO increases

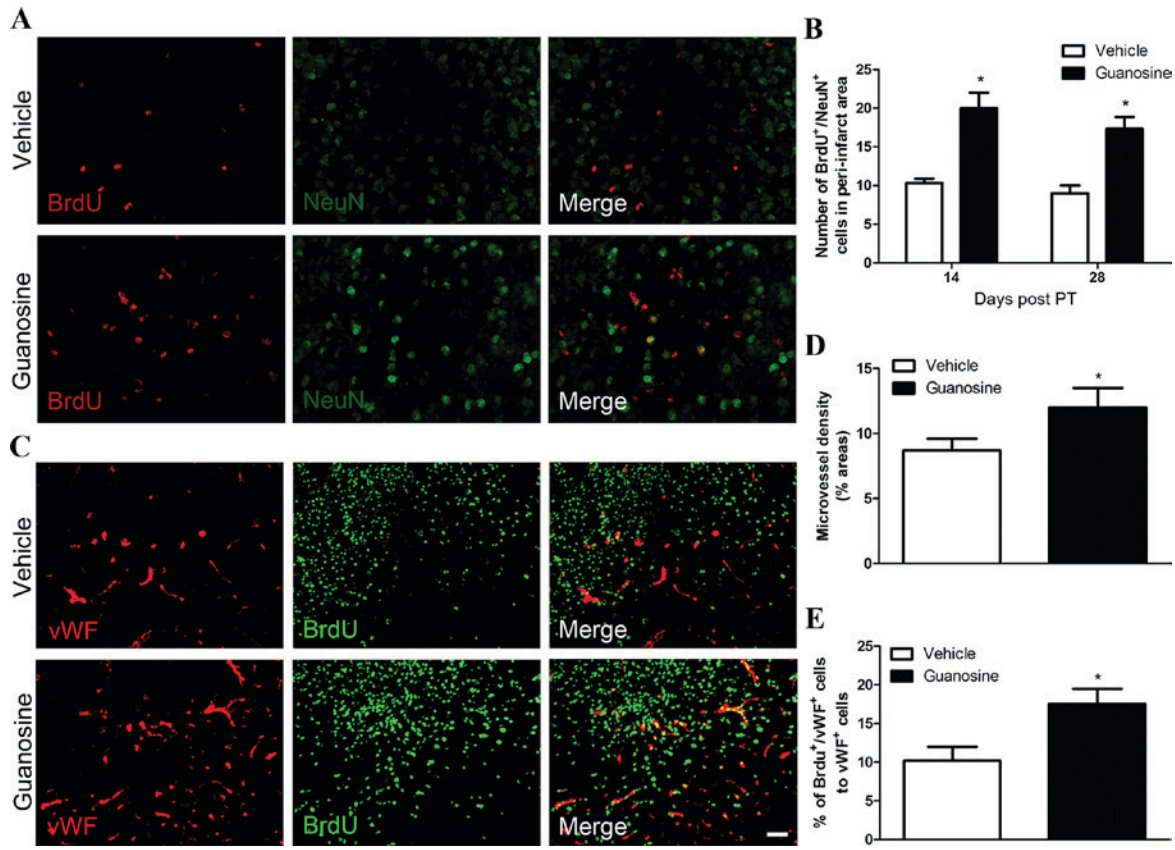

Figure 4. Delayed administration of guanosine increases the number of BrdU$^+$/NeuN$^+$ cells and microvessel density within the peri-infarction region following stroke. (A) Immunostaining of (red) BrdU and (green) NeuN in the peri-infarct region of vehicle- and guanosine-treated mice at day 14 post-stroke. (B) Quantification of BrdU$^+$/NeuN$^+$ cells in the peri-infarct region for each group. (C) Immunostaining of (red) vWF and (green) BrdU in the peri-infarct region of vehicle- and guanosine-treated mice at day 14 post-stroke. (D) microvessel density and (E) percentage of BrdU$^+$/vWF$^+$ cells relative to total vWF$^+$ cells in the peri-infarct region for each group. Data are presented as the mean ± standard error of the mean, n=6 per group. *P<0.05 vs. Vehicle. Scale, 100 μm. BrdU, bromodeoxyuridine; NeuN, neuronal nuclei; vWF, von Willebrand factor.

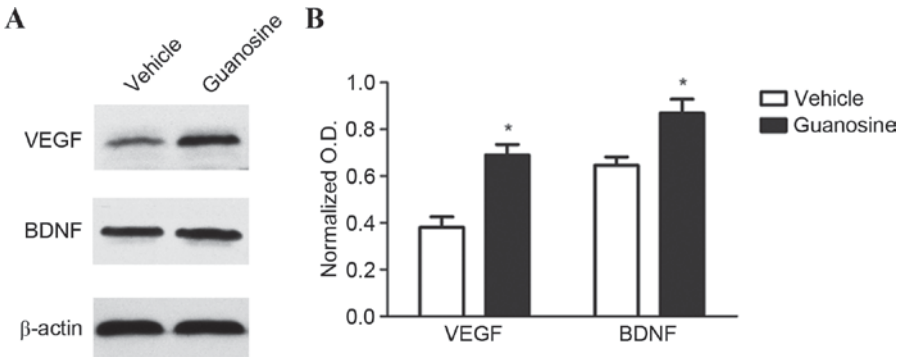

Figure 5. Delayed administration of guanosine increases the expression of VEGF and BDNF following stroke. (A) Representative western blots present levels of VEGF and BDNF in the ischemic brain 28 days following stroke. (B) Quantification (mean optical density, normalized to β-actin) of western blots. Data are presented as the mean ± standard error of the mean, n=6 per group. *P<0.05 vs. Control. VEGF, vascular endothelial growth factor; BDNF, brain-derived neurotrophic factor.

neurogenesis or angiogenesis post-stroke has never been studied, to the best of the authors' knowledge. GUO significantly increased the number of BrdU$^+$ cells in the SVZ and the SGZ, indicating that GUO promotes cell proliferation following stroke. As the number of BrdU$^+$/DCX$^+$ cells increased in the SVZ in GUO-treated mice, GUO enhanced proliferation of endogenous neural progenitor cells. At 14 and 28 days post-stroke, treatment with GUO significantly increased the number of BrdU$^+$/NeuN$^+$ cells in the peri-infarct region, when compared with the vehicle-treated group, suggesting that GUO promoted cell proliferation and the differentiation of new neural progenitor cells into mature neurons within the peri-infarction region. GUO was demonstrated to increase the microvessel density and Brdu$^+$/vwF$^+$ cells in the peri-infarct region, when compared with the vehicle group, indicating angiogenesis post-stroke was enhanced and may contribute to neurological recovery.

Growth and neurotrophic factors have been demonstrated to promote neurogenesis and angiogenesis and improve

neurological function following cerebral ischemia (25,26). Previous *in vitro* studies have repeatedly demonstrated the neurotrophic effects of GUO (27). The present results further suggested that GUO significantly increased BDNF and VEGF levels in ipsilateral brain post-stroke. BDNF and VEGF are two important neurotrophic factors that have multiple effects on neurogenesis and angiogenesis, for example, they stimulate adult neurogenesis and promote migration of new neurons in the SVZ and dentate gyrus (28,29). In addition, the expression of VEGF is associated with an increase in vascular density in the ischemic penumbra (30). Elevated BDNF and VEGF levels may contribute to the enhanced neurogenesis and angiogenesis by GUO. However, the causative link between them has not been investigated, therefore further studies are warranted.

In conclusion, delayed administration of GUO enhances neurogenesis and angiogenesis post-ischemic stroke and increases the expression of BDNF and VEGF. This contributes to improved long-term functional recovery.

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