

Methylprednisolone improves the survival of new neurons following transient cerebral ischemia in rats

Yu hong Jing^{1,2*}, Yi ping Hou^{1,2}, Yan feng Song², and Jie Yin²

¹Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University. P. R. China, *Email: jingyh@lzu.edu.cn; ²Institute of Anatomy and Embryology, School of Basic Medical Sciences, Lanzhou University. P. R. China

Cerebral ischemia induced the proliferation and differentiation of neural stem cells in discrete regions of brain. However, only a small fraction of the neural stem/progenitor cells survives. In this report, the effects of methylprednisolone (MP) on proliferation, differentiation and survival of neural progenitor cells were explored through early MP administration after occlusion of the middle cerebral artery (MCAo). Transient cerebral ischemia was induced by middle cerebral artery occlusion in Sprague Dawley male rats. One hour, 1 day, 3 days, 14 days, and 28 days after MCAo, neurological examination was performed to evaluate the neurological deficit. MCAo rats were randomly divided into two groups, MP-group was injected MP (30 mg/kg, i.p.) at 3 h, 12 h, and 24 h after MCAo, and vehicle group was injected equal saline (i.p.). Animals were sacrificed at 3 days, 14 days, and 28 days after MCAo. MP was found to decrease apoptosis and TNF- α and IL-6 expression at 3 days after MCAo in the ipsilateral striatum. Moreover, MP significantly increased the migrated new neurons (BrdU+/DCX+) and immature neurons (BrdU+/Tuj1+) at day 14 after MCAo in the ipsilateral striatum. Likewise, MP increased the number of mature neurons (BrdU+/MAP2+) at 28 days after MCAo. However, MP did not affect the progenitor cell proliferation (BrdU+ and Nestin+) at day 3 after MCAo in the subventricular zone (SVZ), but improved the neurological deficit at day 1 and day 3 after MCAo. These results indicate that early MP administration can improve the neurological deficit and enhance the survival of new neurons in the ipsilateral striatum by inhibiting apoptosis and downregulation of inflammatory response after transient cerebral ischemia in rats.

Key words: methylprednisolone, stroke, neural stem cell

INTRODUCTION

Following a century of doubt and controversies, the current consensus is that new neurons are generated throughout life from neural stem cells in the subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ) of the dentate gyrus in the brain of the majority of mammalian species, including humans (Zhao et al. 2008, Carpentier et al. 2009, Delcroix et al. 2010; see however Amrein et al. 2007, Bartkowska et al. 2008). Acute brain injury such as cerebral ischemia increases the ratio of neurogenesis in both the SVZ and SGZ of adult rodents (Marti-Fabregas et al. 2010), which suggests the activation of neural stem cells that

migrated into lesion regions may involve in self-repair to compensate for the local cell loss (Jin et al. 2006, 2010). Unfortunately, the majority of new neurons in the adult brain are not preserved. Following ischemic stroke, 80% of new neurons die within 2 to 6 weeks after stroke (Arvidsson et al. 2002, Jablonska and Lukomska 2011), which indicates that new neurons that originated from precursor cells are susceptible to brain injury (Hayase et al. 2009). Accordingly, strategies to enhance the differentiation of progenitor cells into neuroblasts and improve the survival of new neurons after brain injury are beneficial (Ali and Bahbahani 2010).

The survival of new neurons is dependent on the surrounding milieu, which is prominently altered after cerebral ischemia, usually through the accumulation of inflammatory factors, mitotic factors and toxins (Thored et al. 2009, Yoshimura et al. 2001). Although, the mechanism remains unclear, components of the

Correspondence should be addressed to Y.H. Jing
Email: jingyh@lzu.edu.cn

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immune response have recently been proposed to be part of the neurogenic environment that affects progenitor cells proliferation, differentiation and survival. Previous studies have been demonstrated that inflammatory and mitotic factors, including TNF- α and FGF, are increased after cerebral ischemia, which enhances the proliferation of adult neural stem cells *in vivo* and *in vitro* (Wu et al. 2000, Yoshimura et al. 2001, Wiedera et al. 2006). In contrast, apoptotic signals are induced by inflammatory factors and toxins after cerebral ischemia (Broughton et al. 2009), which also induce the die of precursor cells (Saino et al. 2010). Thus, inflammation has a dual effect in determining the fate of neural precursor cells. Presumably, the accumulation of inflammatory factors and toxins causes cell death in the area of the lesion and damages precursor cells in remote areas at the early onset of brain injury. Therefore, early inhibition of the inflammatory reactions may facilitate the survival of new neurons after cerebral ischemia.

Recent studies have been shown that glucocorticoids improve the survival of transplanted cells in the brain by suppressing the rejection and inhibiting the inflammatory response (Khoo et al. 2011). Methylprednisolone (MP), a glucocorticoid clinically used to protect central nerves from acute injury is administered to treat spinal injury and improve the repair after damage (Chang et al. 2009, Tsao et al. 2009). Current studies have also been demonstrated that administration of MP can help protect ischemic brain injury, whose mechanisms have been ascribed to suppress the MCP-1 expression and decrease the aggregation of macrophages in lesion brain regions (Kim et al. 1995). Alternatively, Feng and coauthors (2011) suggested that dexamethasone pretreatment protects brain against hypoxia ischemic injury through the upregulation of vascular endothelial growth factor expression. The fate determination of adult neural stem cells is regulated by variety of endogenous and exogenous factors including inflammation (Mathieu et al. 2010). However, little is known about how the inflammatory response induced by acute cerebral ischemia affects the fate of adult neural stem/progenitor cells in SVZ and striatum, and whether early administration of MP can improve the survival of new neurons. This study investigates an opportunity for anti-inflammatory intervention for treatment of stroke. MP is routinely used in clinic for acute treatment of spinal cord injury, but failed to reveal positive effect in patients with stroke (Gomes et al. 2005). In contrast, several animal studies revealed

that MP can improve an outcome of stroke survivors. Due to failure of neuroprotective drugs in clinical studies, the phenomenon of stroke-induced neurogenesis rises growing interest in the context of target for therapeutic intervention. It is well known that glucocorticosteroids inhibit constitutive neurogenesis (Boku et al. 2010), while this effect was not investigated for stroke-induced neurogenesis yet. Thus this study shows very interesting results that glucocorticosteroid (MP) may actually support stroke-induced neurogenesis, not compromise.

METHODS

Animals and reagents

Seventy-two Adult male Sprague–Dawley (SD) rats (220–250 g) from Lanzhou University Experimental Animal Center were used in this study. The Medical Experimental Animal Administrative Committee of Gansu province approved all experiments. All efforts were made to minimize animal suffering and reduce the number of animals' using. MP was purchased from Pharmacia (Solu-medrol®, Pharmacia, Nerviano, Italy). TUNEL kit was purchased from Roche (Roche, USA). IL-6 and TNF- α ELISA kits were purchased from R&D (R&D, USA). BrdU and diaminobenzidine (DAB) were purchased from Sigma (Sigma, St. Louis, MO). Rabbit polyclonal anti-Nestin, anti-Tuj1, anti-DCX, anti-GFAP and anti-MAP2 were purchased from Millipore (Billerica, MA). Mouse monoclonal anti-BrdU obtained from sigma (Sigma, St. Louis, MO). VECTASTAIN® ABC-alkaline phosphatase Kits (anti-mouse or rabbit IgG) and VECTASTAIN® ABC-peroxidase Kits (anti-mouse, rabbit or goat IgG) and Vector® Blue were purchased from Vector Laboratories, Inc. (Burlingame, CA). Other reagents were from Biotechnology of Shanghai (Shanghai, China).

Cerebral ischemia

Middle cerebral artery occlusion (MCAo) was induced by intraluminal filament method as described previously (Vakili et al. 2007). Briefly, animals were anesthetized with chloral hydrate (360 mg/kg, i.p.) and the right common carotid (CCA) and external carotid artery was exposed. A nylon thread (4–0) was carefully inserted into the internal carotid artery and advanced towards the origin of the middle cerebral artery until a slight

resistance was felt. Such resistance was indication that tip of nylon thread was wedged at the beginning of anterior cerebral artery (20–22 mm from CCA bifurcation), resulting in occlusion of middle cerebral artery. During the process of ischemia, laser Doppler Perfusion Monitor (Periflux system 5000, Terimed AB, Sweden) was used to detect blood flow in the trunk of the middle cerebral artery synchronously. The blood flow dropped to $\geq 60\%$ of the baseline denoting the block of the middle cerebral artery was successful. After 30 minutes of MCAo, reperfusion was accomplished by withdrawing the intraluminal filament. Rectal temperature was measured by a thermometer and maintained at $37 \pm 0.5^\circ\text{C}$ throughout the experiment using an electrical blanket. Animals were then recovered from anesthesia, and kept in single cages for 24 h. Control group, with only incision in their

cervical skin to explore their common artery, were not treated with occlusion of blood flow.

Administration of MP

MCAo rats were randomly divided into two groups, MP-group was injected MP (30 mg/kg, i.p.) at 3 h, 12 h, and 24 h after MCAo according to Betz and colleague’s protocol and modified (Betz et al. 1990), and vehicle group was injected equal saline (i.p.). Animals were sacrificed at 3 days, 14 days, and 28 days after MCAo (Fig. 1A), respectively.

BrdU labeling

For proliferation experiment, 18 rats were intraperitoneally injected with BrdU (50 mg/kg) one time a day at 1 day, 2 days, and 3 days after MCAo and sacrificed

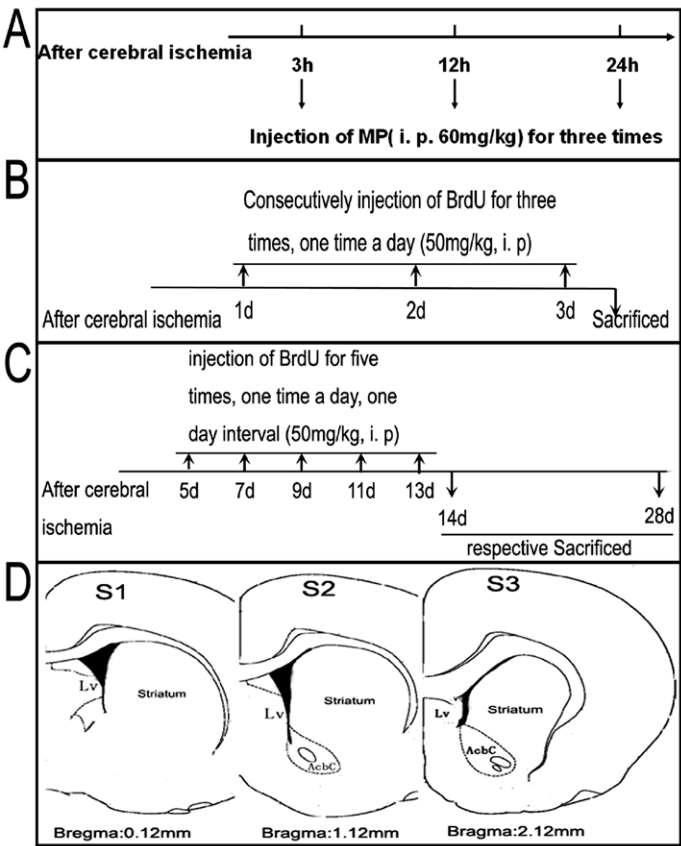


Fig. 1. Schematic diagram for methodologies. (A) Time course of MP administration, MP injection through intraperitoneal at 3 h, 12 h, and 24 h after MCAo. (B) BrdU injection at short survival paradigm was used to analyze progenitor cells proliferation. (C) BrdU injection at long survival paradigm, was used to analyze progenitor cells differentiation. (D) Three section levels each rat were selected at 0.12 mm, 1.12 mm, and 2.12 mm from bregma according to the atlas of rat brain, respectively. (Lv) Lateral ventricle; (AcBc) Accumbens nucleus.

at 3 days after MCAo as shown in Figure 1B. For migration and differentiation experiment, 36 rats were intraperitoneally injected with BrdU (50 mg/kg) at 5 days, 7 days, 9 days, 11 days, and 13 days after MCAo and sacrificed at 14 days and 28 days, respectively, as shown in Figure 1C.

Neurological deficit evaluation

One hour, 1 day, 3 days, 14 days, and 28 days after MCAo, a modified neurological examination was performed to evaluate the neurological deficit in rats (Costa

et al. 2006). Briefly, the scores were: 0 – no apparent neurological deficits (normal); 1 – failure to extend left forepaw; 2 – circling to the left; 3 – falling to the left; 4 – depressed consciousness; and 5 – death. The mean of neurological scores was calculated without cumulative mortality (death was accounted only once). The neurological deficits were assessed in a blinded fashion.

Tissue preparation

Fifty-four rats were sacrificed with overdose of 10% chloral hydrate and transcardially perfused with 0.9%

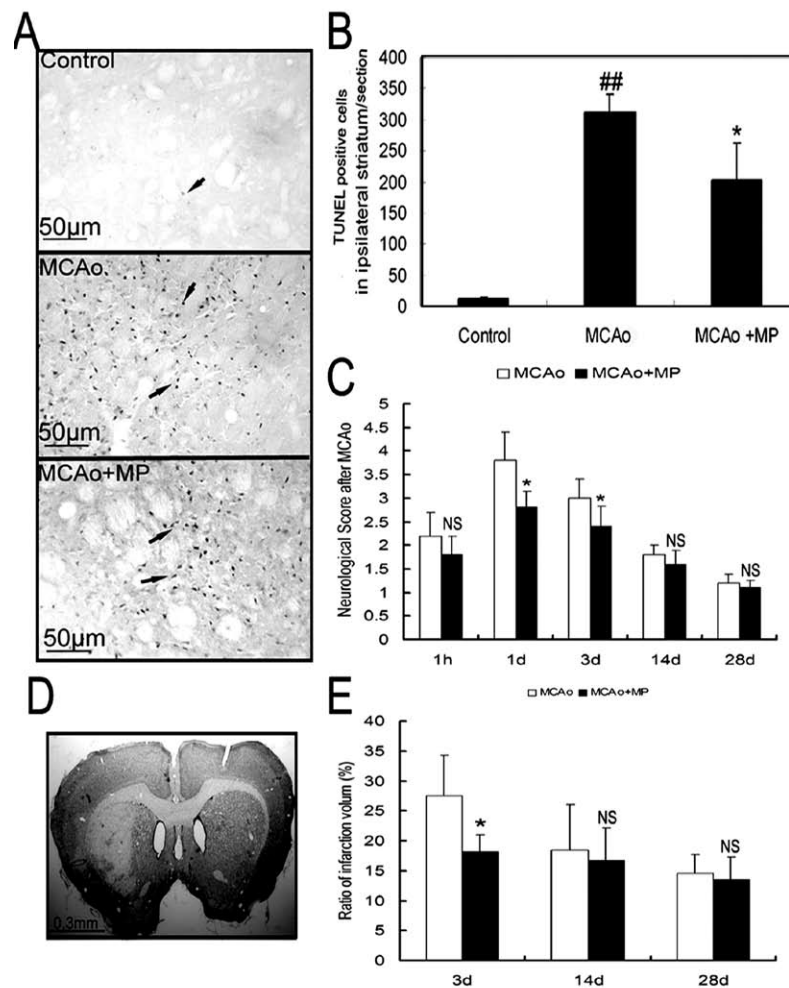


Fig. 2. Effects of MP administration on brain injury after cerebral ischemia. (A) Representative images of TUNEL staining. (B) Three sections each rats at 3 days after MCAo were selected and performed TUNEL assay, and positive cells were counted. (C) The neurological scores for rats were evaluated at 1 h, 24 hours, 3 days, 14 days, and 28 days after MCAo and reperfusion treatment. We used the standard grading score of 0–5 (0: normal, 1: failure to extend left forepaw, 2: circling to the left, 3: falling to the left, 4: depressed consciousness, 5: death). [(D) and (E)] The infarction volume was measured by staining with cresyl violet at 3 days, 14 days, and 28 days after MCAo. The representative image was shown in D, and ratio of infarction volume was shown in E. ^{##} $P < 0.01$ compared with control; ^{*} $P < 0.05$ compared with MCAo; (NS) $P > 0.05$ compared with MCAo ($n=6$).

saline solution followed by 4% ice-cold phosphate-buffered paraformaldehyde (PFA). The brains were then removed and post-fixed in 4% PFA for 12 hours and then immersed sequentially in 20% and 30% sucrose solutions in 0.1 M phosphate buffer (pH 7.4) until they sank. Coronal sections were cut using a freezing microtome (Jung Histocut, Model 820-II, Leica, Germany) at a thickness of 30 μ m at 1.60 to -4.80 mm from bregma and stored at -20°C in cryoprotectant solution.

Measurement of infarct size

Coronal sections were cut serially (25 μ m thickness at 250 μ m intervals) from 4% phosphate-buffered paraformaldehyde (PFA) fixed brains and stained with

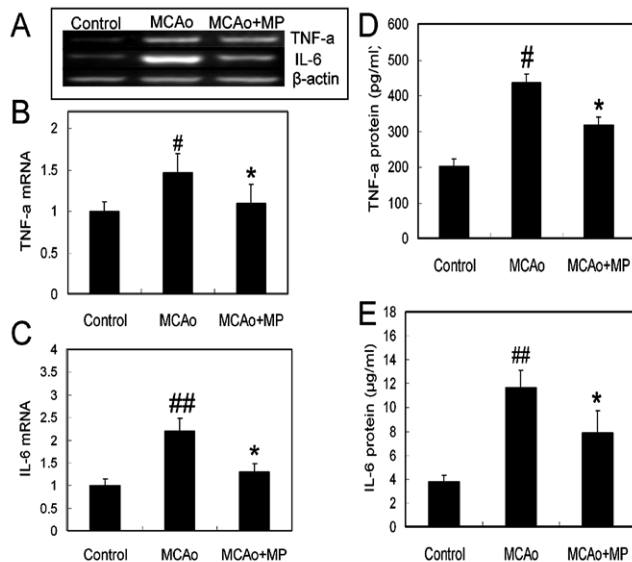


Fig. 3. MP administration decreased high expression of TNF- α and IL-6 following transient cerebral ischemia. Rats at day 3 after MCAo were sacrificed and the ipsilateral striatum were isolated on ice plate. (A) Representative images of three independent experiments for RT-PCR. [(B), (C)] Images were scan and density were measured by image pro-plus software for RT-PCR, and normalized with β -actin. (B) Relative level of TNF- α mRNA in ipsilateral striatum. (C) Relative level of IL-6 mRNA. # $P<0.05$ compared with control; ## $P<0.01$ compared with control; * $P<0.05$ compared with MCAo group ($n=3$). [(D), (E)] The ipsilateral striatum were homogenized and fractioned for analyzing protein levels of TNF- α and IL-6 using ELISA kits, respectively. (D) TNF- α content in ipsilateral striatum. (E) IL-6 content in ipsilateral striatum. # $P<0.05$ compared with control; ## $P<0.01$ compared with control; * $P<0.05$ compared with MCAo ($n=6$).

cresyl violet (CV) to identify viable cells. Infarct areas were measured by an image processing and analysis system (Q570IW, Leica, Germany) using a 1.25 \times objective. Infarct volume, expressed as a percentage of whole-brain volume, was calculated by integration of infarct area on each brain section along the rostra-caudal axis.

TUNEL assay

The rats were sacrificed at 3 days after MCAo, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) assay was performed on three sections selected at 0.12 mm, 1.12 mm, and 2.12 mm from bregma (Fig. 1D) each rat. Sections were treated with 0.2% H₂O₂ for 20 min, rinsed in 0.1 M PBS, and incubated with Proteinase K Solution (contain 200 mmol Tris, pH, 7.4, 0.5mmol EDTA, and proteinase K 1 mg/ml) for 30 min at room temperature. Then the sections were incubated in TUNEL reaction mixture using the In Situ Cell Death Detection Kit, POD (Roche) for 1 h at 37°C, then rinsed in 0.1 M PBS three times for 5 min and incubated in Converter-peroxidase (POD) for 30 min at 37°C, rinsed in 0.1 M PBS three times for 5 min, and color-developed with SIGMA FAST, a diaminobenzidine (DAB) POD substrate.

Semi-quantitative RT-PCR

Nine rats were decapitated and removed the brain at 3 days after MCAo, total RNA was extracted from the ipsilateral striatum using the guanidine isothiocyanate method according to Ribaudo and colleagues (2001). For RT-PCR, 2 μ g of total RNA was reverse transcribed using Moloney Murine Leukemia Virus (M-MLV) reversetranscriptase (Promega, Mannheim, Germany), RNase Inhibitor rRNasin[®] (Promega), dNTP master mix (Invitex, Berlin, Germany) and random hexamer primers (Promega). PCR was carried out using Taq DNA polymerase (Promega), dNTP master mix (Invitex, Berlin, Germany) and the following primers. Primers for rat TNF- α mRNA were as follows: forward, 5'-TCG AGT GAC AAG CCC GTA-3' and reverse, 5'-CAG AGC AAT GAC TCC AAA GTA GAC-3', annealing temperature 52°C, 35 cycles, amplicon size: 447 bp. Primers for rat IL-6 mRNA were as follow: forward, 5'-AAA

ATC TGC TCT GGT CTT CTG-3' and reverse, 5'-GGT TTG CCG AGT AGA CCT CA- 3', annealing temperature 55°C, 35 cycles, amplicon size: 300 bp. Equal equilibration was determined using rat β -actin primers (forward: 5'-TTC TAC AAT GAG CTG CGT GTG G-3', reverse: 5'-ATA CCC AGG AAG GAA GGC TGG AAG-3', annealing temperature 55°C, 25 cycles, amplicon size: 539 bp). PCR products were separated electrophoretically on a 2% agarose gel. Three independent experiments were measured.

ELISA assay

Nine rats were sacrificed at 3 days after MCAo with over dose chloral hydrate and the brain were dissected. The fresh isolated ipsilateral striatum was homogenized with a glass homogenizer in RIPA buffer (pH 7.2, 1 mM PMSF, 1 mg/L of pepstatin A, 1 mg/L of aprotinin and 1 mg/L of leupeptin) and centrifuged at 12 000 g for 20 min at 4°C. The supernatant was then collected and total protein was determined by the Bradford method. The levels of TNF- α and IL-6 in the tissue supernatants were measured using ELISA kits, respectively. The measurements of both TNF- α and IL-6 were performed step by step based on the protocol booklet of the ELISA kits according to the specifications given by the manufacturer.

Immunohistochemistry

For Nestin immunostaining, sections (at 0.12 mm, 1.12 mm and 2.12 mm from bregma each rat, Fig. 1D) were incubated with 0.3% H₂O₂ for 30 min and then placed in blocking buffer containing 10% normal goat serum and 0.3% Triton X-100 in 0.01 M phosphate-buffered saline (PBS, pH 7.2) for 30 min at 37°C and incubated with antibodies against rabbit polyclonal anti-Nestin (1:500) overnight at 4°C. Sections were then incubated with corresponding biotinylated secondary antibodies (1:200, 1 h at 37°C) followed by avidin–biotin–peroxidase (1:200, 1 h at 37°C). Immunoreactivity was visualized with 0.05% DAB as chromogen. Negative controls received the same treatment omitting the primary antibodies and showed no specific staining. For double staining of BrdU and DCX, Tuj-1, GFAP, MAP2, free-floating sections were first incubated with 50% formamide/2 \times SSC (0.3 M NaCl, 0.03 M sodium citrate) and 2 N HCl for DNA denaturation and then rinsed with Tris buffer (0.1 M, pH 7.6) and treated

with 1% H₂O₂ for 10 min. After washing with buffer A (0.1 M Tris buffer/0.1% Triton X-100) and buffer B (0.1 M Tris buffer/0.1% Triton X-100, 0.05% BSA) for 15 min each, sections were placed in 10% goat serum for 1 h at 37°C and incubated with mouse monoclonal anti-BrdU antibody (1:200) overnight at 4°C. After washing, the sections were incubated with biotinylated anti-mouse IgG (1:200) and avidin–biotin–alkaline phosphatase complex (1:200) for 1 h at 37°C. BrdU immunoreactivity was revealed with Vector® Blue. After washing, sections were treated again with primary antibodies against goat polyclonal anti-DCX (1:200), rabbit polyclonal anti-Tuj-1 (1:400), MAP2 (1:200) or GFAP (1:400) overnight at 4°C respectively, then incubated with corresponding biotinylated secondary antibodies and avidin–biotin–peroxidase, then visualized with DAB.

Cell number counting

We performed total cell number counting in a double-blinded fashion. In these experiments, Nestin, BrdU positive cells and BrdU+/Tuj1+, BrdU+/DCX+, BrdU+/MAP2 and BrdU+/GFAP+ double-labeled cells in ipsilateral striatum from three sections (at 0.12 mm, 1.12 mm, and 2.12 mm from bregma, Fig. 1D) each rat was counted under 20 \times objective.

Data quantification and statistical analysis

All data were expressed as mean \pm SEM. Differences between groups were analyzed by ANOVA. The Mann–Whitney *U*-test was used for each evaluation. For other data, unpaired Student's *t* test was used, and *P*<0.05 was considered statistically significant.

RESULT

MP treatment decreased ischemia-induced apoptosis in ipsilateral striatum

Growing evidence has demonstrated that ischemia/reperfusion induces the production of metabolic toxins such as reactive oxygen species, inflammatory factors and excitatory amino acids, which exacerbate brain injury mainly through the activation of apoptotic signals (White et al. 2000). To examine whether MP treatment ameliorates

rates ischemia-induced apoptosis, a TUNEL analysis was performed in selected sections from rats sacrificed at 3 days after MCAo surgery, and the results show that cerebral ischemia increased the number of TUNEL-positive cells in the ipsilateral striatum compared with the controls (Fig. 2A). Furthermore, early MP administration significantly decreased the number of TUNEL-positive cells compared with the untreated ischemia rats (Fig. 2B).

MP-treatment ameliorated the brain injury after MCAo/reperfusion

To estimate if the treatment of MP could result in a reduced functional impairment, the neurological deficits in animals of two groups were blindly evaluated at different time point after MCAo and reperfusion surgery (Fig. 2C). One hour after MCAo and reperfusion, rats of both MCAo and MCAo+MP groups show a similar neuromotor function with mean scores of about 2. During the following 1 day and 3 days after surgery, the neurological deficits of MCAo rats worsened with the increase of scores to 3.7 ± 0.36 and 3.0 ± 0.5 , respectively. While

the administration MP after MCAo resulted in an improvement of the functional outcome with the decrease of scores to 2.6 ± 0.48 and 2.4 ± 0.5 , respectively. These results demonstrated that the neurological function got worse because of focal cerebral ischemia/reperfusion and the extent of functional brain damage was moderately reduced by treatment with MP. The infarction volume was also measured and shown that MP administration significantly decreased the infarction volume (18.2 ± 2.8) compared with untreated rats (27.5 ± 6.7) at 3 days after MCAo. However, there is no significant difference in MP administration and untreated group at 14 days and 28 days after MCAo (Fig. 2D,E).

MP treatment attenuated ischemia-induced inflammatory reactions in the ipsilateral striatum

Inflammatory factors, including IL-6 and TNF- α , induce neuronal apoptosis *in vivo* and *in vitro* (Zhu et al. 2010, Murray et al. 2011). Therefore, whether ischemia induces TNF- α and IL-6 production and the effects of

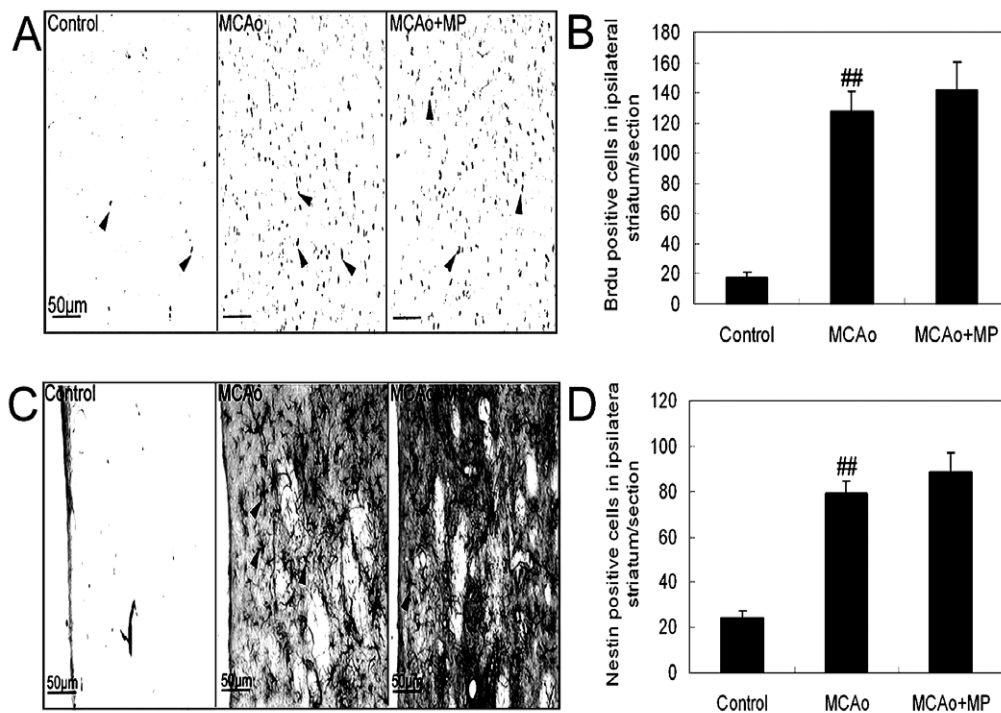


Fig. 4. Effects of MP treatment on ischemia-induced cell proliferation in SVZ at 3 days after MCAo. Representative images of BrdU-immunostaining (A), ischemia-induced increased the numbers of BrdU positive cells, but no significantly changed with MP treatment (B). Representative images of Nestin-positive cells (C), ischemia-induced enhanced the proliferation of progenitor cells (Nestin-positive cells), but no significantly changed with MP treatment (D). Data were reported as mean \pm SEM ($n=6$), ^{##} $P<0.01$ compared with control. (NS) $P>0.05$ compared with MCAo. Arrows indicate immunostaining positive cells.

MP treatment were determined at 3 days after MCAo surgery. Reverse transcription-polymerase chain action (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) methods were used to measure TNF- α and IL-6 expression at the transcriptional and translational levels in the ipsilateral striatum. The level of TNF- α mRNA and protein were elevated in the ipsilateral striatum of the ischemic rats compared with the control group (Fig. 3A, B, D). Similarly, the IL-6 mRNA and protein levels were elevated in the ipsilateral striatum of the ischemic rats (Fig. 3A, C, E). The expression of both TNF- α and IL-6 were decreased in the ipsilateral striatum of the MP treatment group compared with the untreated group after cerebral ischemia.

Effect of MP treatment on ischemia-induced cell proliferation in ipsilateral striatum

To examine the cell proliferation at an early stage, BrdU-labeled methods were used to detect S-phase cells

in the ipsilateral SVZ in ischemic rats at 3 days after MCAo. At early stage, ischemia induced more astrogenesis less than neurogenesis. So, many cells labeled by BrdU are glias. The BrdU-positive cells were increased in the ipsilateral SVZ following cerebral ischemia compared with the control group. No significant difference in the number of BrdU-positive cells were observed between the MP-treated ischemic rats and the untreated controls (Fig. 4A, B). Alternatively, Nestin, a neural progenitor marker (Ernst and Christie 2006), was used to detect the actively proliferating cells in the SVZ. Cells labeled by Nestin were shown the astrocytic morphology. The number of Nestin-positive cells increased in ischemic rats compared with the controls, and no significant difference was observed between the MP treated and untreated ischemic rats (Fig. 4C, D). These results indicate that MP treatment does not affect the ischemia-induced elevation of cell proliferation in the lesion regions. And it should not be connected to neurogenesis, which is most prominent 6–8 days post insult (Janowski et al. 2008).

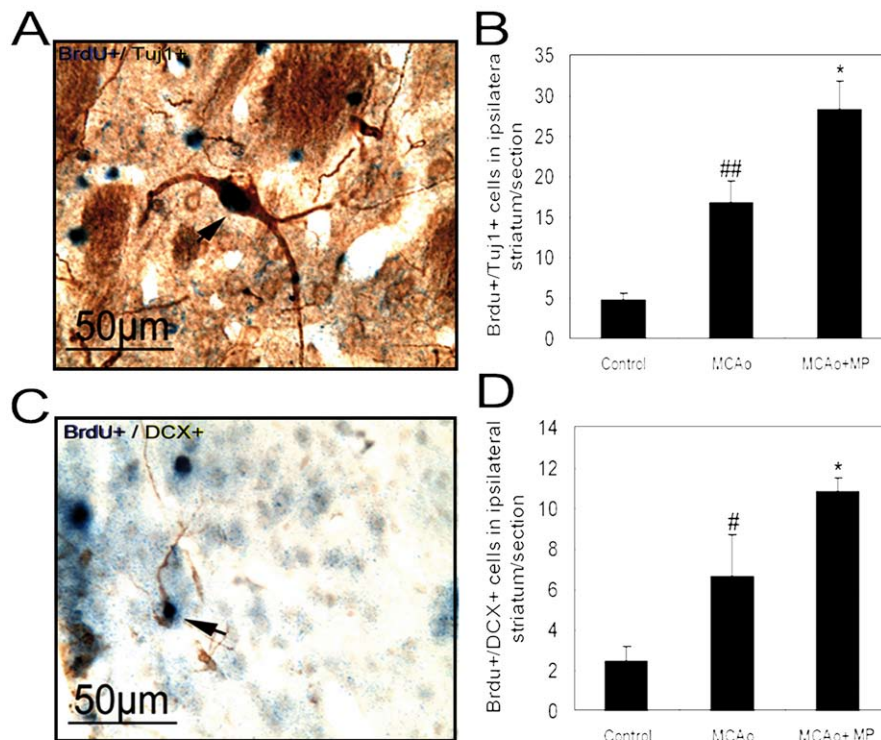


Fig. 5. MP administration improved the survival of new neurons in the ipsilateral striatum at 14 days after transient cerebral ischemia. Double immunostaining with BrdU (blue) and Tuj1 (brown) or DCX (brown) was used to identify immature neurons or migrated neuroblasts in the ipsilateral striatum, respectively. Photographs showed BrdU-labeled cells that co-labeled (arrow) with Tuj1 (A) or DCX (C). Total positive cells were counted in the ipsilateral striatum and shown in (B) (BrdU+/Tuj1+) or (D) (BrdU+/DCX+). MP administration increased the numbers of immature neuroblasts (BrdU+/Tuj1+) and migrated neuroblasts (BrdU+/DCX+). ## P <0.01 and # P <0.05 versus the control. * P <0.05 versus the MCAo (n =6).

MP treatment improved the survival and differentiation of progenitor cell in SVZ after cerebral ischemia

To study further whether MP affects the survival of progenitor cells in the SVZ after prolonged ischemia, double-labeling methods were used to observe and count different cell phenotype cells that originated from progenitor cells in rats at 14 and 28 days after MCAo. The BrdU+/DCX+ positive cells, as migrating-neuroblasts, increased in the MP treated ischemic rats at 14 days after MCAo compared with the untreated rats (Fig. 5A, B). Likewise, the BrdU+/Tuj1+ positive cells, as immature neurons, also increased in the MP-treated ischemic rats at 14 days after MCAo compared with the untreated rats (Fig. 5C, D). At 28 days after MCAo, some rats were sacrificed to detect the mature differentiated cells that originated from the progenitor cells in the ipsilateral striatum. The BrdU+/MAP2+ positive cells, as mature neuron, increased in the MP treated ischemic rats com-

pared with the untreated group (Fig. 6A, B). Additionally, the BrdU+/GFAP+ positive cells, as mature astrocyte, increased in the MP treated group compared with the untreated group (Fig. 6C, D). These findings suggest that MP treatment improves the survival and differentiation of progenitor cells in the SVZ after cerebral ischemia.

DISCUSSION

Cerebrovascular accidents or stroke are one of the most common causes of death and disability in humans. After ischemic brain injury, which causes severe damage to the striatum, new neurons are generated through the proliferation of precursor cells in the SVZ and possibly also in the striatal parenchyma (Im et al. 2010). However, newly proliferated precursor cells are very susceptible to changes in niche surroundings after cerebral ischemia, particularly to the harmful effects of metabolites such as glutamate, calcium or free oxygen radicals. Therefore, many new striatal neurons likely do not survive (Arvidsson et al.

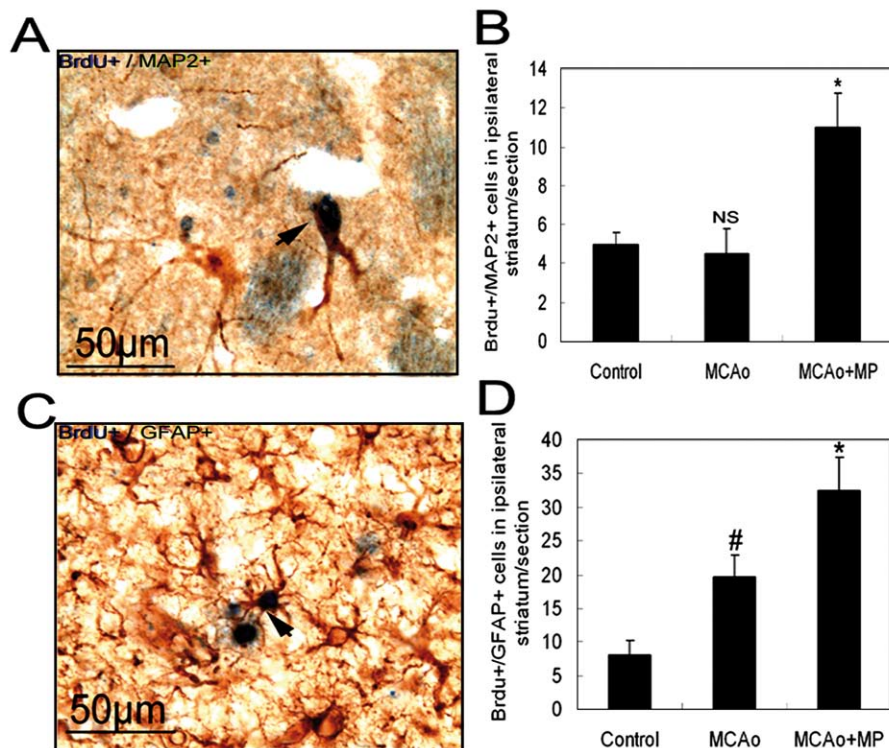


Fig. 6. MP administration enhanced the survival of new neurons in the ipsilateral striatum at 28 days after MCAo. Microphotographs showed Brdu-labeled cells that co-labeled (arrow) with MAP2 (A, as mature neurons) or GFAP (C, as differentiated astrocytes). Positive cells of double-staining were counted in ipsilateral striatum and shown in B (BrdU+/MAP2+) and D (BrdU+/GFAP+). Data indicate that the mature neurons from progenitor cells decreased at 28 days after transient cerebral ischemia, and relatively increased with MP treatment (B). Moreover, BrdU+/GFAP+ double labeled cells still maintained at high level at 28 days after MCAo, and MP treatment also improved their survival (D). * $P < 0.05$ and (NS) $P > 0.05$ versus control ($n = 6$). * $P < 0.05$ compared with MCAo ($n = 6$).

2002). This raises the possibility that treatments that augment the generation or survival of neuroblasts might improve recovery after stroke. Most growth or neurotrophic factors such as BDNF, IGF and VEGF are candidates for improving ischemia-induced neurogenesis (Bath and Lee 2010). Additionally, attenuating ischemia-induced apoptosis is beneficial to the survival of precursor cells, as supported by *in vivo* and *in vitro* studies (Zhang et al. 2006, Luo et al. 2009), which indicates that bcl-2 overexpression or p53 suppression promotes the survival of progenitor cells in the SVZ following ischemic brain injury. The results also demonstrate that early MP administration attenuates ischemia-induced cell apoptosis and increases the survival of new born neurons, as well as enhances their migrating into the lesion regions of the striatum.

MP is commonly given to patients with global brain ischemia, but despite its efficacy, its use has produced mixed conclusion. These drugs alter the host tissue inflammatory responses, including immune reactions and inflammatory responses, which have been implicated as secondary cell death processes in the progression of strokes (Borlongan 2000, Raghavendra et al. 2002, Xia et al. 2004). Both these secondary cell death processes have been shown to mediate the transformation of the ischemic penumbra into an ischemic core (Borlongan 2000, Raghavendra et al. 2002, Xia et al. 2004). The ischemic penumbra, an area populated by viable cells but injured or otherwise destined to die (i.e., apoptotic) without immediate treatment intervention, could become part of the unsalvageable necrotic core (Borlongan et al. 1996). Thus, targeting both immune and inflammatory responses potentially stops the cascade of the pathologic progress. In the present studies, early MP administration effectively protects against brain injury by inhibiting the apoptotic signals in the ipsilateral striatum. Further study indicates that these effects are partly ascribed to reduced TNF- α and IL-6 levels.

Several studies have been indicated that neuroblasts generated after stroke form chains that migrate from the SVZ into the injured striatum (Zhang et al. 2007, Hou et al. 2008). This structural arrangement is remarkably similar to the chain migration of neuroblasts through astrocytic tubes, which is characteristic of the rostral migratory stream (RMS). Lois and others (1996) suggested that the cues induced by ischemic brain injury may redirect some of the SVZ neuroblasts to migrate into the ischemic penumbra (Parent et al. 2002). At 14 days after MCAo, increased numbers of BrdU-DCX double-labeled neurons

were found in the striatum. On the other hand, at 28 days after the MCAo, the numbers of BrdU-MAP2 double-labeled mature neurons has decreased significantly. These results are consistent with previous studies (Alvarez-Buylla et al. 2001), which indicate that ischemia-induced neurogenesis is transient and the fraction of dead striatal neurons that have been replaced by the new neurons is small, only about 0.2% (Arvidsson et al. 2002). Therefore, most of progenitor cells died during their development. Interestingly, MP administration increased the number of BrdU-DCX or BrdU-MAP2 double labeled neurons in the ipsilateral striatum after transient cerebral ischemia, which suggests that MP inhibits the death of new neurons and improves their survival through the suppression of the inflammatory reaction and cell apoptosis. Additionally, the results show that the number of BrdU-GFAP double-labeled cells still remained higher in the ipsilateral striatum of ischemic rats than those of the controls at 28 days after MCAo, which suggests that the progenitor cells in the SVZ may prominently differentiate into glial cells. These results are also consistent with those of previous studies in transient cerebral ischemic models (Jin et al. 2010). Substantial evidence indicates GFAP-expressing progenitor cells as a predominant source of constitutive adult neurogenesis, suggesting that neural stem cells could retain cytological aspect and functions of highly specialized cells in the CNS (Alvarez-Buylla et al. 2001). If the neural stem cells are indeed glialike cells, then the neuronal progenitors would require the down-regulation of glial markers (e.g., GFAP), the appropriate reprogramming of the genetic machinery, and the subsequent determination of lineage and differentiation. If astrocytes or a subset of the astroglial population really have stem cell properties, we need to know more about the regulation of these cells, as the factors necessary to redirect them into neuronal lineage.

Brain injury induces the activation of endogenous neuronal stem cells, which migrate into the regions of brain injury, differentiate into neuronal cells, and integrate into neuronal circuits (Madani et al. 2006). However, whether inflammation regulates neurogenesis remains unclear. Growing evidence indicates that the mediators released by immune cells, such as cytokines and nitric oxide (NO), negatively regulate adult neurogenesis (Vallieres et al. 2002, Monje et al. 2003, Liu et al. 2006). Our current data indicate that inflammatory response was initiated after transient cerebral ischemia and the release of inflammatory cytokines such as IL-6 and TNF- α in the ipsilateral striatum. Numerous *in vitro*

studies have shown that the IL-6 antibodies selectively restore reduced survival of new hippocampal neurons when new neurons co-cultured with microglial cells are activated by LPS, or are exposed to their conditioned medium, which suggests that this effect is likely mediated by IL-6 (Liu et al. 2005, Nakanishi et al. 2007, Cacci et al. 2008). Supporting this concept, transgenic mice with chronic astrocytic IL-6 expression show a substantial decrease in the production of new neurons (Vallieres et al. 2002). Increased TNF- α production by microglial cells during hippocampal inflammation could contribute to the death of new hippocampal progenitor cells (Vezzani et al. 2002). When added to adult hippocampal progenitor cell cultures, TNF- α decreased neurogenesis by 50% (Monje et al. 2003). In the present study, early MP administration significantly inhibited the production of TNF- α and IL-6 following cerebral ischemia. Furthermore, MP treatment enhanced the survival of ischemia-induced neurogenesis in the striatum for a long term. Ischemia also augmented cell proliferation, as shown by the BrdU-labeling or Nestin-marking in SVZ, but MP treatment did not affect their proliferative ability. Together, these imply that the role of MP on the fate of progenitor cells after stroke is mainly attributed to improved survival. Unfortunately, MP improved the neurologic deficit at early stage after ischemia, but lack of effect to long term behavior (up to month). MP positive early behavioral effect may account for anti-inflammatory effect, what may also facilitate stroke-induced neurogenesis. The lack of long-term positive effect (up to one month) is in-line with clinical studies and may be related to the complex situation *in vivo*. Presumptively, the effect of MP on neurogenesis is not sufficient to produce positive behavioral effect. Additionally, one month is too short period to reveal positive behavioral effect, because newly formed neurons may need much more time to integrate into brain neuronal circuitries.

In the future study, we need to further elucidate the fate of progenitor cells in SVZ, and to clarify the therapeutic period of MP. Also we need to answer whether the effects of MP on survival of new neurons dependents anti-inflammation or other effects.

CONCLUSION

Although, the present experiment could not elucidate the concrete molecular mechanisms underlying MP regulation of ischemia-induced neurogenesis, early MP administration clearly improves the neurological deficit

and enhances the survival of new neurons following a stroke by inhibiting the cell apoptosis and inflammation.

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