

## Original Article

# Catalpol enhances neurogenesis in the SVZ in a rat model of strokes

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**Abstract:** Recent studies have shown that catalpol provides neuroprotective effects. It also increases vascular endothelial growth factor and erythropoietin in stroke-affected brains. It remains unclear, however, whether catalpol can enhance neurogenesis after strokes. In the current study, rats were administered catalpol and bromodeoxyuridine (BrdU), following permanent occlusion of the middle cerebral artery (pMCAO). Neurogenesis in the subventricular zone was observed. Results showed that catalpol increased the number of new immature neurons (BrdU<sup>+</sup>-Nestin<sup>+</sup> and BrdU<sup>+</sup>-Tuj-1<sup>+</sup>) at day 7 after strokes. Moreover, catalpol treatment increased Tuj-1, MAP2, and brain-derived neurotrophic factor expression levels. It also increased the ratio of Bcl-2/Bax. Behavioral assessment demonstrated that catalpol improved neurological outcomes. The laterality index decreased significantly following treatment with catalpol, as well. Present results indicate that catalpol enhances neurogenesis and the survival of new neurons. Therefore, it may be a potential strategy for treatment of strokes.

**Keywords:** Catalpol, neurogenesis, Bcl-2/Bax, SVZ zone, permanent occlusion of the middle cerebral artery (pMCAO)

## Introduction

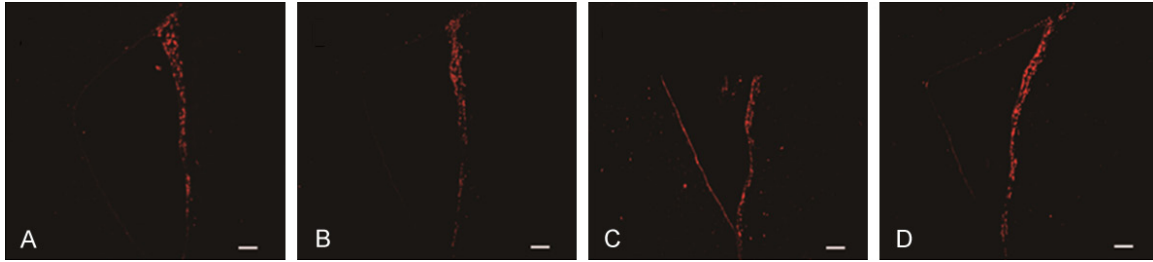
Strokes are one of the most common causes of disability and death in humans. Despite improvements in treatment methods, targeting ischemic pathophysiology, in recent years, neuroprotective therapy for strokes remains limited to a narrow time-window. Tissue plasminogen activator (TPA) is, at present, the only approved treatment for strokes. It is only effective during the first 3-6 hours immediately after the stroke. It also produces side effects, such as cerebral hemorrhaging [1]. Endogenous neural stem cells are potential therapeutic targets, as they produce new neurons after strokes. Adult neurogenesis occurs throughout the lifetime in restricted regions of the brain, including in the dentate gyrus of the hippocampus, subventricular zone (SVZ), and olfactory bulbs [2].

The SVZ of the lateral ventricle contains neural stem cells and progenitor cells that generate

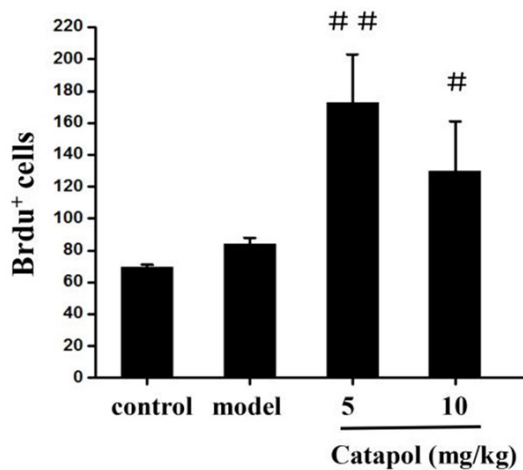
neuroblasts. Ischemic strokes induce neurogenesis in the SVZ. These cells migrate to the boundary of the ischemic lesion site [3]. Recent evidence has demonstrated that enrichment of the vascular niche in the environment is involved in proliferation, differentiation, migration, and survival of neural progenitor cells after strokes [4]. Stimulation of the endogenous brain repairing processes, including angiogenesis and neurogenesis, holds promise as a potential novel strategy, restoring neural function after strokes [5].

Catalpol, an important iridoid glycoside compound purified from *Rehmannia glutinosa Libosch*, can upregulate vascular endothelial growth factor (VEGF) and erythropoietin (EPO) expression. This promotes angiogenesis via the Janus kinase 2 (Jak2)/signal transducer and activator of transcription 3 (Stat3) pathways in brains suffering from strokes. This ameliorates the edema of endothelial cells of the brain capillar-

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**Figure 1.** Catalpol-enhanced neurogenesis, expressed as the density of cells labeled with BrdU, was recorded at 7 days after strokes in the subventricular zone (n=6). BrdU, bromodeoxyuridine. A. Sham group; B. Model group; C. Catalpol 5 mg/kg group; D. Catalpol 10 mg/kg group. BrdU (50 mg/kg i.p.) was administered twice daily. (magnification,  $\times 100$ ; scale bar, 100  $\mu\text{m}$ ).



**Figure 2.** Number of bromodeoxyuridine-positive cells in the subventricular zone of the rat brain ipsilateral to middle cerebral artery occlusion at 7 days post-stroke (n=6). \* $p < 0.05$  vs. model, \*\* $p < 0.01$  vs. model.

ies [6, 7]. Moreover, catalpol enhances brain derived neurotrophic factor (BDNF) expression in normal and post-stroke brains [8]. However, it remains unclear whether catalpol can enhance neurogenesis after strokes.

Addressing this problem, the current study focused on potential neurogenic effects of catalpol. The current study examined its mechanisms and evaluated anti-apoptotic effects on new-formed neurons. This study also assessed neuronal functional recovery after treatment with catalpol in a rat model of permanent middle cerebral artery occlusion (pMCAO) [6, 7]. Results suggest that catalpol may enhance neurogenesis and promote neuronal stem cell differentiation into neurons by suppressing downregulation of Bcl-2 and upregulation of Bax, enhancing BDNF following strokes [9].

### Materials and methods

#### Animals and pMCAO model

All experimental procedures were performed, as described previously [6, 7]. Adult male Sprague-Dawley rats were housed under natural illumination with food and water available *ad libitum*, according to National Guidelines for the Care and Use of Laboratory Animals. All experiments met ethical requirements for experimental animals. The current study was approved by the Ethics Committee of the College of Pharmaceutical Sciences and Chinese Medicine, Southwest University. Strokes were induced in the rats by electrocoagulation of the right middle cerebral artery, as previously described [6, 7]. The rats were prescreened, selecting those that met phenotypic criteria based on Bederson et al. [10].

#### Groups and drug treatment

A total of 36 animals were randomly divided into 4 groups, including the pMCAO group (model), sham operated group (Control), 5 mg/kg catalpol-treated group, and 10 mg/kg catalpol-treated group (n=9 in each). Each of the 9 rats were subjected to behavioral assessment. A total of 6 rats were used to analyze neurogenesis with bromodeoxyuridine (BrdU) and immunofluorescent staining. The remaining 3 rats were used for Western blotting. Catalpol (>98% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). It was dissolved in physiological saline (KeLun Co., Chengdu, China). Catalpol was administered 6 hours after pMCAO, then given daily for 7 days at 5 or 10 mg/kg (0.5 or 1 mg/mL). The sham-operated group and vehicle group received

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**Table 1.** Corner test scores of in each group at different time points after strokes (n=9)

Group	Day 1	Day 3	Day 7
Sham	0.55±0.04	0.53±0.08	0.52±0.10
Model	0.97±0.05**	0.92±0.10**	0.87±0.08**
Catalpol (mg/kg) 5	0.92±0.07	0.82±0.08#	0.68±0.08##
10	0.95±0.04	0.87±0.08	0.75±0.05#

\*\*P<0.01 vs. sham; ##P<0.01, #P<0.05 vs. model.

physiological saline treatment at 1 mL/100 g bodyweight via intraperitoneal injections.

### Behavioral assessment

Neurobehavioral performance was evaluated at days 1, 3, and 7 after pMCAO. Bederson scores were used to evaluate stroke modeling success. They were used daily to examine neurological function, based on a 4-point scale [10]: 0) No apparent deficits; 1) Contralateral forelimb flexion; 2) Lowered resistance to lateral pushing without circling; and 3) As grade 2, with circling ipsilateral to the pMCAO.

### Corner rotation testing

The animal were placed facing a corner with a 30-degree angle formed by vertical boards. The boards at the corner did not make contact, with a gap of 0.5 cm. This encouraged the animals to move deep into the corner and rear and to turn around when contacting the boards with the whiskers. Ten trials on each day of two test days were conducted for reliable preinjury assessment of turning learning. Animals with scores that did not change with repeated testing were removed from analyses. Every complete 180° turn to the left or right (relative to the rat) during the first 5 minutes of the corner test was scored. Incomplete turns were not included in statistics analysis (Figure 2). Laterality index (LI) was calculated by dividing the number of right turns by the total number of left and right turns [11, 12].

### BrdU injections

In the current study, 5-Bromo-2'-deoxyuridine (BrdU, Sigma, USA) was used to examine the effects of catalpol on neurogenesis in rats suffering from strokes. Labeling the dividing cells, three animals in each group received BrdU (50 mg/kg) twice a day, with a 12-hour interval between doses for 3 days, starting

from the 4<sup>th</sup> day after the stroke [13]. This dose of BrdU was selected to saturate mitotic cell labeling, as previously determined [14]. These rats were sacrificed on day 7 after the stroke. BrdU-labeled cells were then counted to assess new neurons [15]. Rats used for Western blotting were treated with the same amount of physiological saline at the same time.

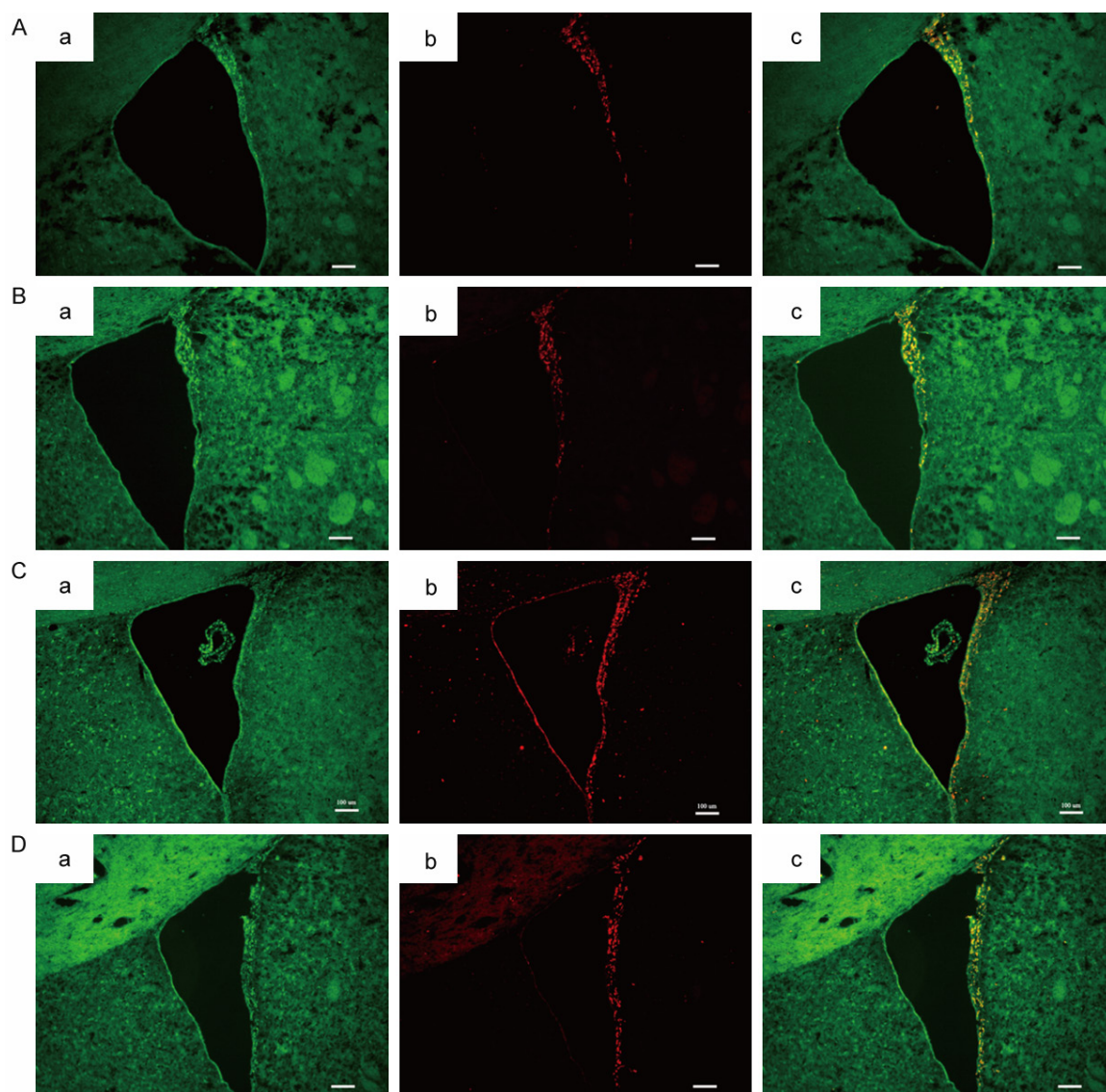
### Tissue preparation

A total of 7 days after the operation/stroke, the rats were sacrificed with an overdose chloral hydrate. They were transcardially perfused with a 0.9% NaCl solution to rinse out the blood. This was followed by perfusion with 250 mL of 4% formalin (4°C) to fix the brain tissues. After extraction from the skull, the brains were post-fixed in a 4% formalin solution. They were subsequently cut into 10 µm coronal sections on a cryostat (Leica) for immunohistochemistry. Ipsilateral ischemic cortices (0.1 g per brain) in each group were weighed for Western blotting [7, 8].

### Immunohistochemistry

Immunofluorescent staining of brain tissues was performed on the coronal sections (10-µm thin), as previously described [7, 8]. Primary antibodies included mouse anti-BrdU (1:300; Proteintech), mouse or rabbit anti-tubulin III (TuJ-1, 1:200; Cell Signaling Technology, USA), and rabbit anti-Nestin (1:50; Proteintech, Wuhan, China). This was followed by secondary Cy3-conjugated goat anti-mouse IgG (1:100; Proteintech, Wuhan, China) and FITC-conjugated goat anti-rabbit IgG (1:100; ZSGB Biotech. Co., Beijing, China) antibodies. The immunostained cells were observed under a Nikon microscope and documented with a Nikon digital camera (Nikon Corporation, Japan). Immunofluorescence staining for Nestin or TuJ-1 (green) and BrdU (red) was visualized and documented with a confocal microscope (Leica, Germany). The number of double-stained cells and intensity levels of staining were analyzed with Image Pro Plus version 6.0 software (Media Cybernetics) at the ischemic boundary zone. Five fields from each slice were randomly selected for treatment-blinded scoring and analysis. Negative controls omitted the primary antibody from similarly treated adja-

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**Figure 3.** Catalpol-enhanced neurogenesis, expressed as the density of cells labeled with Nestin (green) and BrdU (red) immunostaining in the subventricular zone of the rat brain ipsilateral to middle cerebral artery occlusion at 7 days after the procedure in each group (magnification,  $\times 100$ ; scale bar,  $100\ \mu\text{m}$ ;  $n=6$ ). (A) Sham group; (B) Model group; (C) Catalpol  $5\ \text{mg/kg}$  group; (D) Catalpol  $10\ \text{mg/kg}$  group; (a) Labeled with Nestin (green), (b) BrdU (red) and (c) co-Nestin and BrdU (yellow).

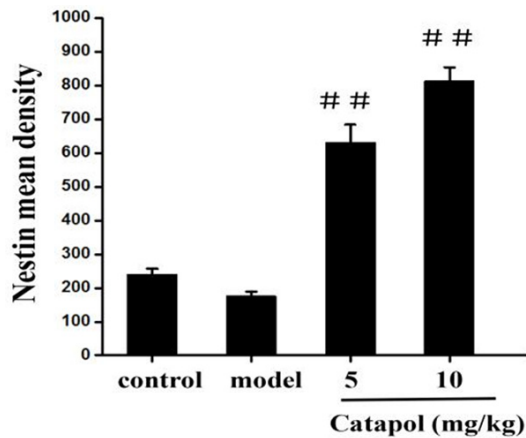
cent sections. Each experiment was performed three times.

### Western blotting

Western blotting and cellular fractionation were performed, as previously described [6]. Briefly, the peri-ischemic brain cortex was lysed on ice in a lysis buffer [50 mM Tris-HCl (pH 8.2), 0.5 M saccharose, 10 mM HEPES (pH 7.9), 1.5 mM  $\text{MgCl}_2$ , 10 mM KCl, 1 mM EDTA, 10% (v/v) glycerin, 1 mM DTT, 1 mM PMSF,  $10\ \mu\text{g/mL}$  Apro-

tinin, and  $5\ \mu\text{g/mL}$  Leupeptin from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany)]. After centrifugation at  $16,000\ \text{r/min}$  for 10 minutes, protein concentrations in the cleared lysate were determined using Bradford assays. Lysate samples containing  $50\ \mu\text{g}$  protein were fractionated by 10% SDS-polyacrylamide gel electrophoresis (90 V for 15 minutes, then 110 V for 1 hour) and electroblotted onto PVDF membranes ( $4^\circ\text{C}$ , 100 V for 90 minutes). The following antibodies were used at indicated concentrations: Mouse anti-tubulin III

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**Figure 4.** Mean density of Nestin protein expression in the subventricular zone of the rat brain ipsilateral to middle cerebral artery occlusion after 7 days in each group (n=6). <sup>#</sup><sup>#</sup>p<0.01 vs. model.

(TuJ-1, 1:200; Cell Signaling Technology), rabbit anti-Nestin (1:50; Proteintech), Bcl-2 (1:300, Bioss Beijing, China), Bax (1:2000, Proteintech), TrkB (1:2000, Proteintech), p-TrkB (1:2000, Proteintech), GAPDH (Bioworld Technology, 1:3000, Louis Park, USA), horseradish peroxidase (GenScript, 1:5000, Nanjing, China), and anti-mouse horseradish peroxidase (GenScript, 1:5000). Immunoreactive bands treated with ECL kit (GeneCopoeia, USA) were digitally scanned using the Tanon MP system. They were quantified using a Tanon Gis gel imaging system (Tanon, Shanghai, China). NADPH was used as an internal control for all Western blots.

### Statistical analyses

Data are expressed as mean  $\pm$  SEM. Present data was analyzed with one-way analysis of variance (ANOVA) and post-hoc testing (Bonferroni's method). SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for analyses. P<0.05 indicates statistically significant differences.

## Results

### *Catalpol improves neurological outcomes in the corner turn test*

Analyzing video data from corner turn testing, turning behavior was quantified. Corner turn test results were analyzed using the laterality index (LI). As shown in **Table 1**, sham group rats typically left the corner without demon-

strating a side preference. The LI decreased from days 1 to 7. Rats with induced strokes commonly left the corner towards the non-impaired (left) body side (P<0.05 vs. sham) at each time point (days 1, 3, and 7). The LI decreased significantly following treatment with catalpol at both concentrations (5 and 10 mg/kg) (P<0.05 vs. model). Spontaneous recovery was observed at days 1-7 after focal ischemia (**Table 1**).

### *Catalpol significantly improves BrdU-labeled cell counts in the SVZ*

Induction of strokes increased the number of BrdU labeled cells in the ipsilateral SVZ at 7 days after induction, compared with the corresponding area in the sham group. The difference was not significant (P>0.05). However, both catalpol doses (5 and 10 mg/kg) significantly increased the number of BrdU-labeled cells, compared with the model group (P<0.05). More BrdU positive cells were observed in the corpus striatum adjacent to the SVZ area. This was especially obvious in the 5 mg/kg catalpol group. Present data suggests that treatment with catalpol may increase cell proliferation in the SVZ (**Figures 1, 2**).

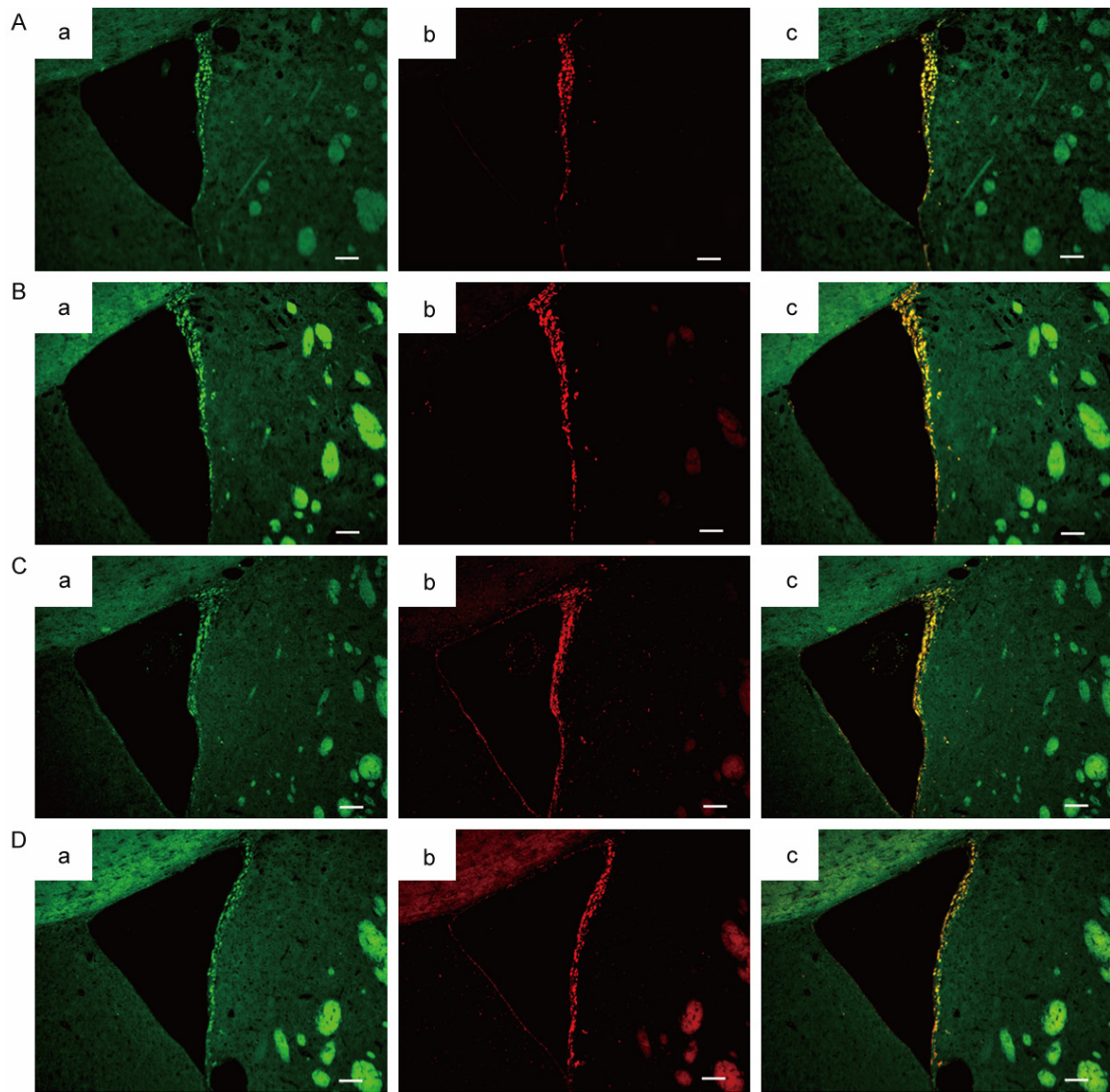
### *Catalpol significantly increases nestin and BrdU co-expression in the SVZ of rat brains ipsilateral to strokes*

Nestin was used as a marker of neuronal progenitor cells. Treatment with catalpol (5 and 10 mg/kg) significantly increased the number of nestin and BrdU-immunoreactive cells in the ipsilateral SVZ, compared with the model group. Catalpol also significantly enhanced Nestin + BrdU co-expression in the SVZ (P<0.01; **Figures 3, 4**).

### *Catalpol significantly increases the number of cells with Tuj-1 and BrdU co-expression in the SVZ of rat brains ipsilateral to strokes*

Newly-formed immature neurons were detected in the SVZ area by double-labeling with BrdU and Tuj-1 at 7 days after the procedure. Induction of strokes increased the counts of BrdU/Tuj-1 double-positive newly-formed neurons, while no new immature neurons were observed in the sham operation group. Interestingly, in both the low concentration and high concentration catalpol group (5 and 10 mg/

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**Figure 5.** Catalpol significantly increases the number of cells with Tuj-1 and BrdU co-expression in the SVZ of the rat brain ipsilateral to stroke. Tuj-1 (magnification,  $\times 100$ ; scale bar,  $100\ \mu\text{m}$ ;  $n=6$ ). (A) Sham group; (B) Model group; (C) Catalpol  $5\ \text{mg/kg}$  group; (D) Catalpol  $10\ \text{mg/kg}$  group; (a) Labeled with Tuj-1 (green), (b) BrdU (red) and (c) co-Tuj-1 and BrdU (yellow).

kg), treatment increased the number of BrdU-Tuj-1 double-positive cells. No differences were found between the two concentrations of catalpol (Figures 5, 6).

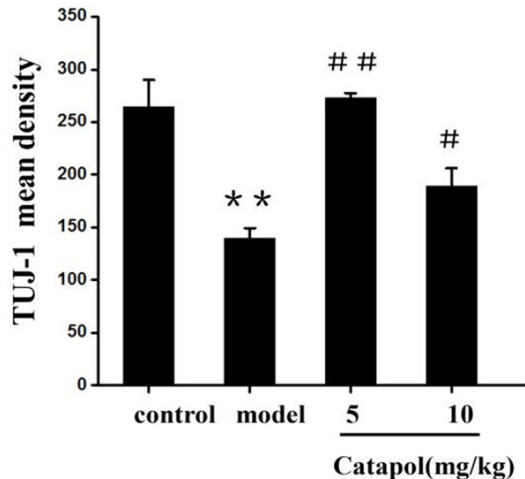
Since Tuj-1 can be used to label new immature neurons and Map-2 can label mature neurons, Tuj-1 and Map-2 expression levels, assessed by Western blotting, may indicate the survival of neurons in the cortex of rat brains surrounding ipsilateral infarction. Results showed that catalpol, at both concentrations, upregulated Tuj-1 ( $189.23\pm 4.28$ ,  $273.03\pm 17.05$  vs.  $139.25\pm 9.98$ ) and Map-2 expression ( $P<0.01$ ;

$1.67\pm 0.07$ ,  $1.69\pm 0.15$  vs.  $0.89\pm 0.04$ ), compared with the stroke model group. In contrast, there were no significant differences between 5 and 10 mg/kg catalpol groups (Figure 7).

### *Catalpol upregulates the ratio of Bcl-2/Bax in vivo*

To determine whether catalpol provides anti-apoptotic effects on new neurons, sections from rats euthanized at 7 days post-stroke were tested for Bcl-2 and Bax by Western blotting. The Bcl-2/Bax ratio increased significantly following treatment with catalpol at 5 and 10

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**Figure 6.** Mean density of Tuj-1 protein in the sub-ventricular zone of the rat brain ipsilateral to middle cerebral artery occlusion at 7 days after the procedure ( $n=6$ ). \* $p<0.05$  vs. model, ## $p<0.01$  vs. model. \*\* $p<0.01$  vs. control.

mg/kg ( $0.88\pm 0.06$ ,  $1.05\pm 0.06$ ;  $P<0.01$  vs. model,  $0.46\pm 0.09$ ). Results suggest that catalpol may decrease the rate of apoptosis of newly-formed neurons in the ischemic striatum of adult rat brains at 7 days after MCAO (Figure 7).

### *Catalpol increases expression of BDNF, but not TrkB and p-TrkB*

To determine whether catalpol increases expression of BDNF and its receptors, the current study examined the effects of catalpol on BDNF, TrkB, and p-TrkB protein expression using Western blotting. Neural cortices of rats surrounding the ischemic core were examined after 7 days. Immunoblot analysis showed that strokes reduced BDNF expression and catalpol, at 5 and 10 mg/kg ( $1.02\pm 0.05$ ,  $1.04\pm 0.05$  vs. model,  $P<0.01$ ), significantly stimulated BDNF expression, compared with the model group ( $0.89\pm 0.05$ ). Regarding the p-TrkB/TrkB protein ratio, surprisingly, no significant differences were observed in any of the groups after permanent ischemia ( $P>0.05$ ) (Figure 8).

### Discussion

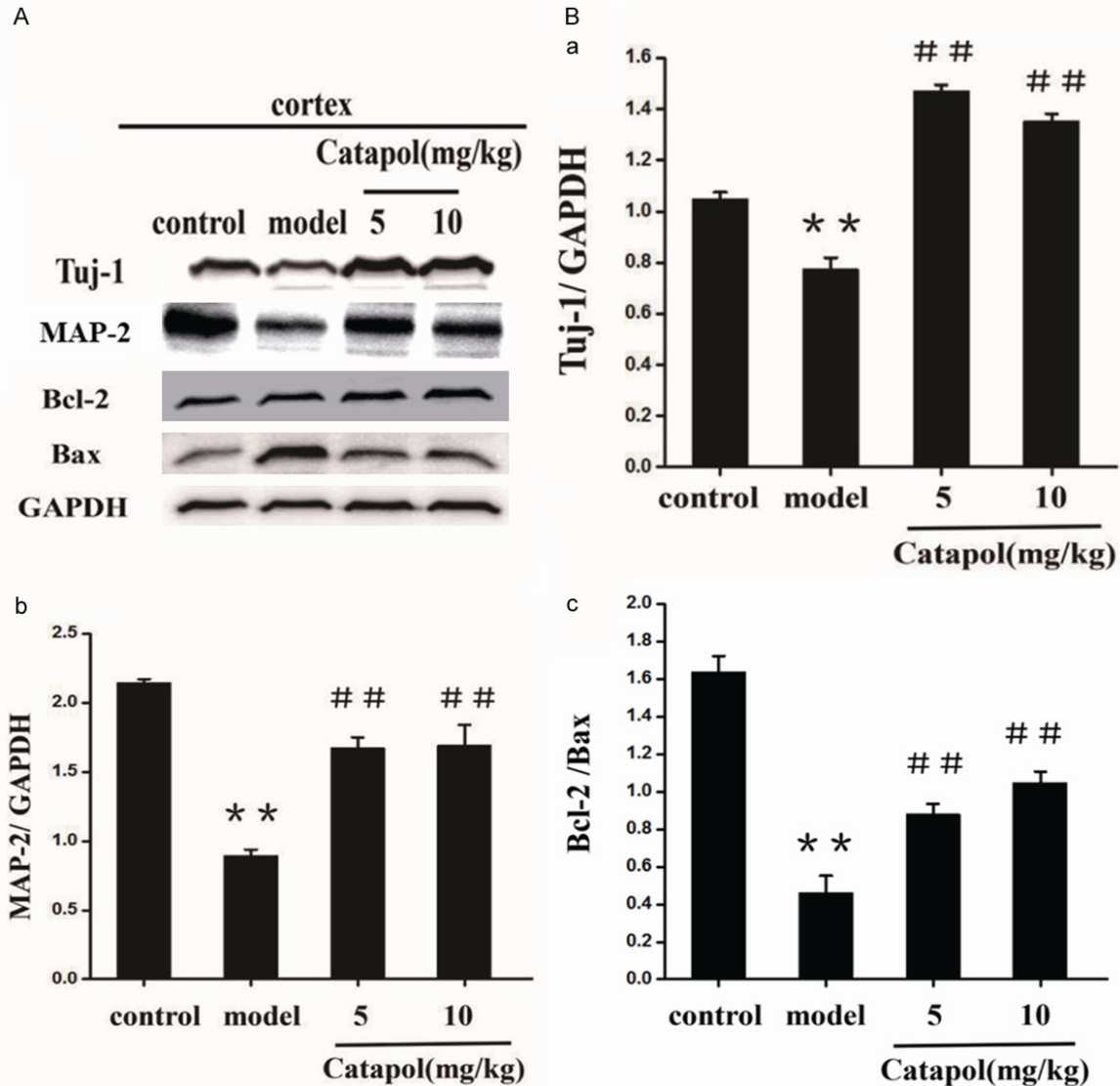
The present study provides novel insight into the roles of catalpol in neuroprotection through modulating adult neurogenesis. It has been reported that catalpol improves angiogenesis in stroke-afflicted brains, efficiently improving post-stroke neurological function in rats [6, 7]. The current study confirmed that catalpol pro-

motes neurogenesis at 7 days after experimentally-induced strokes, improving neurological function recovery (Table 1). Catalpol promoted post-stroke cell proliferation in the SVZ region (Figures 1, 2) and upregulated co-expression of a marker of cell proliferation and a marker of neural stem/progenitor cells (BrdU and nestin, respectively) in the SVZ (Figures 3, 4). After a stroke, neuroblasts in the SVZ migrate toward the ischemic boundary regions of the striatum via a chainlike phenomenon [16]. The current study showed that the BrdU<sup>+</sup>/Tuj1<sup>+</sup> double-labeled cell number was increased in both the SVZ and ischemic boundary regions after treatment with catalpol (Figures 5, 6). Taken together, results suggest that a proportion of neuronal stem cells differentiated into neurons and that catalpol enhanced this differentiation.

Previous evidence has indicated that >80% of new neurons die during the 7-day time interval after a stroke. Only 0.2% of dead striatal neurons are replaced by new neurons within 6 weeks after ischemia [17, 18]. Therefore, a reasonable strategy for stroke recovery may be applied based on increasing neurogenesis, promoting the survival of new neurons and promoting the connection with existing neurons *in situ* [19, 20].

Map2 is a marker of mature neurons. Although the source of these cells was not investigated in the present study, these neurons survived in the cortex after strokes. Tuj1 (Figures 5-7; an immature neuron marker) and Map2 (a mature neuron marker; Figure 7) expression was increased after treatment with catalpol, compared with untreated mice, as assessed by immunohistochemistry and Western blotting.

Most neuroblasts die primarily from apoptotic mechanisms. Only a small population of newly generated neurons survive after strokes [18, 19]. Bcl-2, an inhibitor of apoptosis, together with Bax, a promotor of apoptosis, regulate cell fate and survival. Mounting evidence has suggested that Bcl-2 expression enhances neurogenesis and inhibits the apoptosis of new neurons in normal or ischemic adult rat brains [21, 22]. To investigate potential anti-apoptotic effects of catalpol, Bcl-2 and Bax expression levels were examined. Results showed that induction of strokes promoted Bax expression and decreased Bcl-2 production, while catalpol reversed this phenomenon and increased the



**Figure 7.** Expression of Tuj1, Map-2, Bcl-2/Bax protein, and their density analysis in the cortex of a rat brain surrounding the ischemic core 7 days after procedure assessed by western blot (n=3). Map, mitogen-activated protein. A. Expression of Tuj1, Map-2, and Bcl-2/Bax protein by Western blot. B. Represent density analysis of the Tuj1, Map-2, and Bcl-2/Bax, respectively. Results are normalized by GAPDH. \*\**p*<0.01 vs. control; ##*p*<0.01 vs. model.

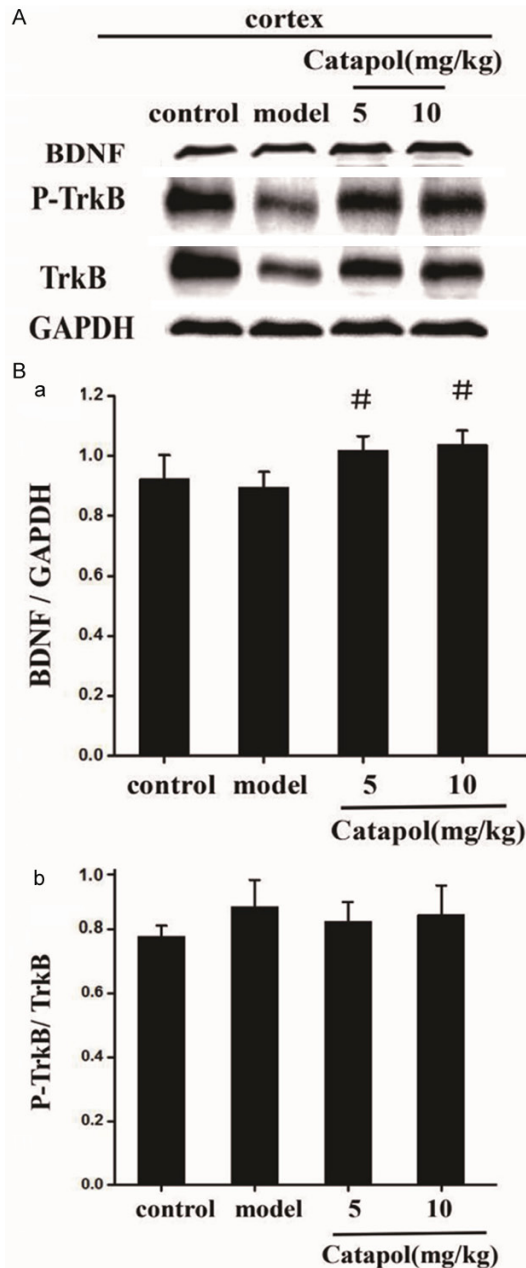
Bcl2/Bax ratio (**Figure 7**). Together with the improved recovery of neurobehavioral function, catalpol enhanced neurogenesis and prevented the death of new neurons by inhibiting apoptosis.

Although the mechanisms of these apoptotic events are unclear, exposure of newly-formed neurons to the detrimental environment of severely damaged tissues, with a lack of trophic support and connections, may play a role in the pathogenesis. Various neurotrophic and growth factors, such as BDNF, EPO, and VEGF, can stimulate neurogenesis [8, 23, 24] and

serve as potent anti-apoptotic factors. Combining previous results and present results (**Figure 8**), catalpol promotes BDNF expression [8] and increases EPO and VEGF secretion [6]. This may provide the necessary support for newly generated cells to survive, eventually leading to improved cell integration within the brain tissue. Unexpectedly, catalpol increased expression of BDNF, but not TrkB or p-TrkB (**Figure 8**). These mechanisms require further research in the future. Finally, in accord with the beneficial molecular and cellular alterations induced by catalpol, this study also demonstrated functional neurological recovery, according to cor-



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**Figure 8.** Expression and density analysis of BDNF/TrkB protein in the cortex of rat brains surrounding the ischemic core 7 days after procedure (n=3). BDNF, brain derived neurotrophic factor; TrkB, neurotrophic tyrosine kinase receptor type 2. A. Expression of BDNF, TrkB, and p-TrkB protein by Western blot. B. Represent density analysis of the BDNF, p-TrkB/TrkB, respectively. Results are normalized by GAPDH. <sup>#</sup>p<0.05 vs. model.

ner turn testing. This test is a sensitive model used to detect functional outcomes after strokes, especially concerning coordination of sensory input, motor function, and balance [26].

Although spontaneous recovery after focal ischemia was observed within 7 days post-stroke, catalpol treatment improved functional outcomes. The side preference and LI decreased significantly after treatment with 5 or 10 mg/kg, compared with the model at each timepoint examined.

In summary, the current study showed that catalpol not only promotes neurogenesis in the SVZ, but also enhances neurological function recovery *in vivo*. The drug also improves neuronal survival by suppressing downregulation of Bcl-2 and upregulation of Bax induced by strokes. Collectively, these results suggest that treatment of stroke-induced rats with catalpol can promote neurogenesis and block the ischemia-induced apoptosis of newly-formed neurons by regulation of Bcl-2 family members. This could be beneficial for neurological function recovery. These findings may be relevant in improving therapeutic interventions for strokes by enhancing neurogenesis, a critical process for functional restoration after ischemia.

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### Disclosure of conflict of interest

None.

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