Role of Wnt/β-catenin in the tolerance to focal cerebral ischemia induced by electroacupuncture pretreatment

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A B S T R A C T

Previous studies have demonstrated that pretreatment with electroacupuncture (EA) elicits rapid tolerance to focal cerebral ischemia and that Wnt/β-catenin plays an essential role in cell survival and proliferation. In the present study, we investigated the role of Wnt/β-catenin in EA pretreatment-induced neuroprotection. Two hours after EA pretreatment, focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) for 2 h. Neuronal survival, cell apoptosis, and the Garcia neurological deficit scores were evaluated 24 h after reperfusion. Moreover, learning and memory deficits were assessed 24 h after reperfusion using the Morris water maze test. Finally, the expression of β-catenin and the B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) ratio were investigated in the presence and absence of the Wnt/β-catenin antagonist Dickkopf-1 (Dkk-1), which was administered 30 min before MCAO. We observed that EA pretreatment significantly increased the neuronal expression of β-catenin in the hippocampus 24 h after reperfusion. Moreover, EA pretreatment improved the neurological outcomes, decreased neuronal loss, inhibited apoptosis, and reversed learning and memory deficits following reperfusion. These beneficial effects of EA were attenuated by Dkk-1, which effectively reversed the expression of β-catenin. Furthermore, the administration of a Wnt/β-catenin agonist upregulated the expression of β-catenin and the Bcl-2/Bax ratio. These results suggest that Wnt/β-catenin plays a role in the protective effects of EA pretreatment against cerebral ischemia, thus providing evidence of a novel mechanism underlying EA-pretreatment-induced rapid tolerance to focal cerebral ischemia.

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1. Introduction

Stroke is one of the leading causes of death and disability worldwide (Liu et al., 2015). Cerebral ischemia triggers a cascade of pathological events that result in irreversible neuronal injury within minutes of stroke onset (Wang et al., 2015). Although substantial progress has been made towards understanding the mechanisms underlying ischemic stroke over the past few decades, there is still a significant need for effective therapies that target acute stroke (Wu et al., 2012). To date, thrombolysis is the only Food and Drug Administration-approved medical therapy for the treatment of patients with acute ischemic stroke. However, only a small number of patients benefit from this therapy because of its narrow therapeutic window (3.0–4.5 h after symptom onset) and incompatibility with various co-morbid medical conditions (Fletcher et al., 2013). Therefore, the research and development of new treatments for stroke are significant challenges for scientists.

Ischemic tolerance is an endogenous protective mechanism wherein sub-lethal ischemia induces neuroprotective processes that can counteract lethal ischemic injury. In the brain, this phenomenon can also be induced by pretreatment with a sub-lethal stimulus other than ischemia (Wang et al., 2005). Electroacupuncture (EA), originating from Chinese traditional medicine, combines traditional Chinese acupuncture with modern electrical stimulation techniques; this method is effective at preventing ischemia/reperfusion (I/R) injury in the brain. For instance, in a recent study, the administration of EA pretreatment for 30 min at

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the Baihui acupoint (GV 20) 2 h prior to focal cerebral ischemia was found to induce ischemic tolerance in rodent models (Zhou et al., 2013). Other studies have demonstrated that the mechanisms underlying EA pretreatment-induced ischemic tolerance are associated with the endocannabinoid system (Wang et al., 2009), while the neuroprotective effects induced by EA pretreatment may involve modulation of the adenosine A1 receptor (Wang et al., 2005). However, the mechanisms responsible for such effects following EA preconditioning remain unclear.

Wnt proteins are evolutionarily conserved secreted glycolipoproteins that play important roles in nervous system development. In the presence of Wnt signals, β-catenin is stabilized and translocated to the nucleus, where it interacts with the T cell factor/lymphoid enhancer factor family of transcription factors to induce alterations in gene expression (Baarsma et al., 2013). Recent studies have suggested that Wnt pathway stimulation induces a neurogenic environment that is primed for neuronal differentiation and survival after ischemic injury (Shruster et al., 2012), while inhibition of the canonical Wnt signaling pathway is required for inducing ischemic and excitotoxic neuronal death (Mastroiacovo et al., 2009). Accordingly, the inhibition of Wnt signaling also suppresses neuron formation and learning in vivo (Shruster et al., 2012; Cho et al., 2013) reported an association between alterations in β-catenin expression in the hippocampal CA1 region and early dendritic damage following transient cerebral ischemia. Moreover, evidence exists to suggest that Wnt/β-catenin signaling pathway activation in the liver could alleviate I/R injury via the amelioration of oxidative stress and the inhibition of proinflammatory cytokine release (Li et al., 2015). Vigneron et al. (2011) reported that the Wnt pathway is involved in the cardioprotective processes against lethal ischemia following ischemic pretreatment, providing further evidence that Wnt signaling is a good potential therapeutic target for acute stroke. Furthermore, the glycolgen synthase kinase 3β/β-catenin signaling pathway reportedly modulates the extent of I/R injury in the central nervous system (Yan et al., 2015), while a recent study suggested that the mechanisms underlying the neuroprotective effects of acupuncture in Alzheimer’s disease (AD) are associated with the regulation of β-catenin expression in the Wnt signaling pathway (Zhou et al., 2014).

Based on these findings, the current study aimed to investigate the potential involvement of the Wnt/β-catenin pathways in EA pretreatment-induced ischemic tolerance by using a rat middle cerebral artery occlusion model.

2. Materials and methods

2.1. Animals

The experimental protocol used in the current study was approved by the Ethics Committee for Animal Experimentation at Wenzhou Medical University and was conducted according to the Guidelines for Animal Experimentation of Wenzhou Medical University. Male Sprague–Dawley rats (280–320 g) were obtained from the Laboratory Animal Center of Silaike in Shanghai, China and housed in a controlled environment (12-h light/dark cycle; 21 ± 2 °C; humidity of 60–70%) for at least 1 week prior to drug treatment or surgery. Animals received standard lab food and water ad libitum.

2.2. Experimental protocols

2.2.1. Experiment I

To confirm the potential therapeutic effects of EA pretreatment in I/R injury and determine the roles of the Wnt/β-catenin signaling pathways, rats were randomly assigned to one of five groups: control, sham, middle cerebral artery occlusion (MCAO), sham electroacupuncture (SEA), and EA. The MCAO group underwent MCAO; the sham group underwent surgery without MCAO; the SEA group received acupuncture without electrical stimulation, and then underwent MCAO 2 h after EA pretreatment; and the EA group received EA pretreatment for 30 min, and then underwent MCAO 2 h after EA pretreatment. The number of viable neurons in the pyramidal layer of the medial CA1 region was assessed using Nissl staining and immunofluorescence (n = 6–8). β-catenin protein expression was determined by western blot analyses (n = 10) and immunohistochemistry (n = 6–8).

2.2.2. Experiment II

To assess the effects of Wnt/β-catenin inhibition on ischemic tolerance following EA pretreatment, rats were randomly assigned to six groups: sham, MCAO, EA + MCAO, Dickkopf-1 (Dkk-1) + MCAO, EA + Dkk-1 + MCAO, and EA + phosphate-buffered saline (PBS) + MCAO. Animals in the sham, MCAO, and EA + MCAO groups received the treatments described above in Section 2.2.1. The Dkk-1 + MCAO group received an intracranial injection of the Wnt/β-catenin antagonist Dkk-1 30 min prior to MCAO. The EA + Dkk-1 + MCAO group received EA pretreatment for 30 min, followed by an intracranial injection of Dkk-1 30 min prior to MCAO; and the EA + PBS + MCAO group received EA pretreatment and a sterile PBS intracranial injection 30 min prior to MCAO. Twenty-four hours after reperfusion, neurological scores were evaluated and the expression of β-catenin and the B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) ratio were evaluated using western blot analyses (n = 10). The number of viable neurons in the pyramidal layer of the medial CA1 region was assessed using Nissl staining (n = 6–8).

2.2.3. Experiment III

To assess the effects of EA pretreatment on the restoration of cognitive function following I/R brain injury, rats were randomly assigned to six groups: sham, MCAO, EA + MCAO, Dkk-1 + MCAO, EA + Dkk-1 + MCAO, and EA + PBS + MCAO groups (n = 12). Each group received the treatments described above in Section 2.2.2. The Morris water maze (MWM) was used to evaluate cognitive function, wherein learning and memory functions were assessed.

2.2.4. Experiment IV

To assess whether administration of the Wnt/β-catenin agonist lithium chloride (LiCl) replicates the beneficial effects of EA pretreatment, rats were randomly assigned to five groups: sham, MCAO, EA + MCAO, LiCl + MCAO, and normal saline (NS) + MCAO (n = 10). Animals in the sham, MCAO, and EA + MCAO groups received the treatments described above in Section 2.2.1. The LiCl + MCAO group received an intraperitoneal injection of LiCl at a dose of 1.0 mEq/kg every 12 h for 7 days prior to MCAO, while the NS + MCAO group received the same volume of NS. Twenty-four hours after reperfusion, the Bcl-2/Bax ratio and β-catenin expression levels were determined using western blot analyses.

2.3. EA pretreatment

EA pretreatment was performed as described previously (Zhou et al., 2013). Briefly, after 12 h of fasting, the rats were anesthetized with intraperitoneal 10% chloral hydrate (350 mg/kg). Oxygen was administered via a facemask at a flow rate of 1.0 L/min. The Baihui acupoint (GV20), located at the intersection of the sagittal midline and the line linking the two ears, was stimulated for 30 min at an intensity of 1 mA and a frequency of 2/15 Hz. EA was performed using an HANS-200A EA instrument (Model No. 200110510586; Nanjing Jisheng Medical Technology Co., Ltd.,...
2.2. Antibodies

Antibodies were used in the current study: anti-β-catenin (H-102; 1:200; Santa Cruz Biotechnology), rabbit polyclonal anti-Bax antibody (1:1000 dilution; Abcam, Cambridge, MA, USA), and anti-Bcl-2 antibody (1:1000 dilution; Abcam, Cambridge, MA, USA), and anti-Bax antibody (1:1000 dilution; Abcam).

2.4. Transient focal cerebral ischemia

Two hours after EA pretreatment, animals were anesthetized with intraperitoneal 10% chloral hydrate (350 mg/kg). Oxygen was administered via a facemask at a flow rate of 1.0 L/min. Focal cerebral ischemia was induced by MCAO using the intraluminal filament technique, as described previously (Geng et al., 2015). Regional cerebral blood flow was monitored using a transcranial laser Doppler flow meter (PeriFlux5000; Perimed AB, Sweden). MCAO was considered sufficient if the regional cerebral blood flow demonstrated a sharp decrease to 20% of the baseline (pre-ischemic) level; if not, the animal was excluded. Reperfusion was achieved by withdrawing the suture after 2 h of ischemia, and then we sutured the wounds. The pericranial temperature was monitored and maintained at 37.0–37.5°C by surface heating or cooling during surgery, until the rats recovered from anesthesia.

2.5. Neurobehavioral evaluation

Twenty-four hours after reperfusion, an observer who was blind to the animal groupings assessed the rats using the Garcia test, which is a neurological examination that utilizes an 18-point scale (Garcia et al., 1995). The assessed items included spontaneous activity, vibrissae touch, side-stroking, limb symmetry, climbing, and forepaw outstretching.

2.6. Western blot analysis

To investigate alterations in β-catenin, Bcl-2, and Bax expression, rats were decapitated under deep anesthesia, and the hippocampus was rapidly dissected and frozen at −80°C until further use. The hippocampus was homogenized in a radioimmunoprecipitation assay lysis buffer (Beyotime, Nantong, China) with a complete protease inhibitor cocktail (Roche Diagnostics Ltd., Mannheim, Germany). Protein levels were measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Aliquots of protein extracts were separated using gel electrophoresis and transferred to polyvinylidene fluoride membranes. Blots were blocked in 5% milk, Tris-buffered saline, and Tween 20 and incubated with primary antibodies overnight at 4°C. Subsequently, the samples were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:5000 dilution; Beyotime). The following primary antibodies were used in the current study: anti-β-catenin (H-102; rabbit polyclonal antibody (1:800 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bcl-2 antibody (1:100 dilution; Abcam, Cambridge, MA, USA), and anti-Bax antibody (1:1000 dilution; Abcam).

2.7. Immunohistochemistry

Brains were removed 24 h after reperfusion. Rats were perfused with 4% paraformaldehyde via the left cardiac ventricle. Paraffin-embedded sections (3 μm thick) were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were treated with 10 mmol/L citrate buffer (pH 6.0) and heated in a microwave for 14 min for antigen retrieval. The slides were allowed to cool for 30 min in the same solution at room temperature and were subsequently washed in PBS. The brain sections were incubated overnight with rabbit polyclonal anti-β-catenin (H-102; 1:200; Santa Cruz Biotechnology) and mouse monoclonal anti-neuronal nuclei (NeuN; MAB377X; 1:100; Millipore, MA, USA). This was followed by incubation with appropriate fluorescent (BS11502; 1:100; Bioworld, USA) or biotinylated secondary antibodies (PV6001; ZSGB-Bio, Beijing, China).

2.8. Histological and quantitative analyses

Dissected brains were prepared as described above (Section 2.7), and sections (3 μm) were processed for staining with Cresyl violet (Nissl staining). To assess the extent of hippocampal injury, the number of surviving neurons in the pyramidal layer of the medial CA1 region was counted under a light microscope (BX60; Olympus, Tokyo, Japan) at 20× magnification. Round neurons similar to those observed in sections from control animals were considered viable.

2.9. Drug treatments

The recombinant human Dkk-1 protein (R&D Systems, MN, USA) was dissolved in sterile PBS at a concentration of 0.1 μg/μL. One microliter of the solution was injected 30 min prior to MCAO using a Hamilton microsyringe (#80135; Hamilton, Reno, NV, USA). Animals received 10% chloral hydrate (350 mg/kg, intraperitoneal) anesthesia and the recombinant human Dkk-1 protein solution was injected into the right CA1 region of the hippocampus at the following stereotaxic coordinates: anterior-posterior = −4.80 mm, medial-lateral = 3.20 mm, and dorsal-ventral = −3.2 mm from bregma (Paxinos and Watson, 1986). The vehicle group received the same volume of sterile PBS. The β-catenin protein levels, cognitive function, number of viable neurons, and Bcl-2/Bax ratio were evaluated 24 h after reperfusion. LiCl (L4408; Sigma–Aldrich, USA) was injected intraperitoneally at a dose of 1.0 mEq/kg every 12 h for 7 days prior to induction of cerebral ischemia. The vehicle group received the same volume of NS. The Bcl-2/Bax ratio and β-catenin expression levels were determined by western blot analyses.

2.10. MWM test

The MWM test was used to evaluate the effects of EA pretreatment on MCAO-induced learning and memory deficits. This involved the use of a large circular pool (height, 60 cm; diameter, 100 cm) filled with water (23 ± 1°C) that contained a small circular platform (height, 29 cm; diameter, 40 cm). The pool was divided into four quadrants, with the abovementioned escape platform placed in one of the quadrants, referred to as the target quadrant. The inner surface of the pool and platform were painted black, and the platform was placed at the midpoint of the target quadrant and submerged approximately 1.0 cm below the surface of the water. The MWM test included an acquisition trial and a probe trial. The acquisition trial was performed to assess learning and memory formation. Rats (n = 12) were trained for six blocks on the MWM (three trials per block) 24 h after reperfusion, with a 30-min rest period between trials. At the start of all trials, the rats were placed in the water so that they were facing the maze wall at different positions. They were then allowed to swim until they found the platform, where they remained for 15 s. The rats were guided to the
platform if they failed to locate it within 1 min, with a maximum latency of 60 s. The escape latency (i.e., the time required to locate and climb onto the platform) was recorded by a video-tracking system (CG-400 Image Acquisition System; Institute of Materia Medica, Chinese Academy of Medical Sciences, Shanghai, China). The probe test (60 s) was initiated 1 h following the completion of the last trial, with the platform removed. Swimming parameters, including swimming speed, time spent in the target quadrant, and swimming track, were recorded.

2.11. Statistical analysis

Statistical analysis was performed using SPSS 15.0 for Windows (SPSS Inc. Chicago, IL). All data except the Garcia neurological deficit scores are presented as the mean ± the standard error of the mean; the Garcia neurological scores are expressed as medians with ranges and were analyzed using the Kruskal–Wallis H test, followed by the Nemenyi test. The escape latency was analyzed using a multivariate analysis of variance (ANOVAs) with block as the dependent variable, group as a covariate; this was followed by Fisher’s least significant difference test. Other analyses were performed using one-way ANOVAs with Fisher’s protected least significance difference test. A P-value of <0.05 was considered statistically significant.

3. Results

3.1. EA pretreatment was neuroprotective against cerebral I/R injury

The current experiment confirmed the findings of previous reports (Zhou et al., 2013) by identifying a distinct therapeutic benefit of EA pretreatment on brain I/R injury. Neuronal cell death was evaluated 24 h after reperfusion in the EA + MCAO and MCAO groups. NeuN staining demonstrated a significant increase in the number of viable pyramidal neurons in the hippocampal CA1 region in the EA + MCAO group compared to the number in the MCAO group (P < 0.05; Fig. 1).

3.2. EA pretreatment upregulated β-catenin expression after reperfusion

As shown in Fig. 2A and B, the β-catenin protein expression level 24 h after reperfusion increased significantly in the EA + MCAO group compared to the level in the MCAO group (P < 0.05). Immunohistochemical analysis showed that there were relatively low levels of β-catenin constitutive expression in the MCAO group, while a substantial increase in β-catenin immunoreactivity was detected in the EA + MCAO group 24 h after reperfusion (Fig. 2C). No difference in the β-catenin protein expression levels was observed between the MCAO and SEA + MCAO groups.

3.3. Dkk-1 inhibited the EA pretreatment-induced upregulation of β-catenin expression

The increase in β-catenin expression that was induced by EA pretreatment was significantly inhibited by Dkk-1 administration (0.1 μg/μL) 30 min prior to focal cerebral ischemia (EA + Dkk-1 + MCAO vs. EA + MCAO, P < 0.05). However, Dkk-1 application did not affect β-catenin expression when administered alone, as no difference in the expression level was observed between the Dkk-1 + MCAO and MCAO groups. Similar results were observed for the EA + MCAO and EA + PBS + MCAO groups (Fig. 3).

3.4. Dkk-1 attenuated the neuroprotective effects of EA pretreatment

Twenty-four hours after reperfusion, the Garcia neurological deficit scores of the EA + MCAO group demonstrated significant improvement compared with the scores of the MCAO group (P < 0.05). Furthermore, Dkk-1 reversed the beneficial effects of EA pretreatment (EA + Dkk-1 + MCAO vs. EA + MCAO, P < 0.05), but did not exert an effect when administered alone. The neurological scores were similar for the EA + PBS + MCAO and EA + MCAO groups, and no significant differences were observed among the MCAO, Dkk-1 + MCAO, and EA + Dkk-1 + MCAO groups (Fig. 4A).

EA pretreatment significantly attenuated neuronal loss in the CA1 region of the hippocampus, a feature that was not observed in the MCAO group (EA + MCAO vs. MCAO, P < 0.05). Dkk-1 inhibited the beneficial effects of EA (EA + Dkk-1 + MCAO vs. EA + MCAO, P < 0.05). No significant differences in the number of viable neurons were detected between the MCAO and Dkk-1 + MCAO groups (Fig. 4B and C).

As shown in Fig. 4D and 24 h after reperfusion, the Bcl-2/Bax levels in the hippocampus were found to be higher for rats in the EA + MCAO group than they were for rats in the MCAO group (EA + MCAO vs. MCAO, P < 0.05). Dkk-1 significantly suppressed the EA pretreatment-induced increases in the Bcl-2/Bax levels (EA + Dkk-1 + MCAO vs. EA + MCAO, P < 0.05), with no difference in the ratio being observed between the Dkk-1 + MCAO and MCAO groups.

![Fig. 1.](image)

Fig. 1. Effects of electroacupuncture (EA) pretreatment on neuronal survival (n = 6–8) after ischemia/reperfusion injury. (A, B) Nissl staining of the hippocampus 24 h after reperfusion. Cell counting shows a significant decrease in the number of viable neurons in the hippocampal CA1 region for the middle cerebral artery occlusion (MCAO) group, while this number is significantly increased in the EA + MCAO group. (C) NeuN immunofluorescence. The arrows indicate the loss of immunofluorescence. *P < 0.05 vs. control group, **P < 0.05 vs. the MCAO group.
3.5. Dkk-1 prevented the EA pretreatment-induced restoration of cognitive function

The efficacy of EA pretreatment in restoring the cognitive function of rats following I/R brain injury was evaluated using the MWM test. As displayed in Fig. 5, spatial learning and memory functions were more severely affected in the MCAO group, while EA pretreatment produced an increase in cognitive function after reperfusion compared to the groups that did not receive EA pretreatment. During the acquisition trials, all rats displayed a progressive decrease in the escape latency. Multivariate ANOVA comparisons revealed significant main effects for group (P < 0.05), but not for the group × swimming speed interaction (P > 0.05). Fisher’s least significant difference test revealed reduced escape latencies in the EA + MCAO group compared to in the MCAO group in all acquisition trials (all P < 0.05). These findings were reversed by Dkk-1 (EA + Dkk-1 + MCAO vs. EA + MCAO, P < 0.05; Fig. 5A).

The probe test revealed a significant difference between the EA + Dkk-1 + MCAO and EA + MCAO groups with regard to the swimming speed (P < 0.05; Fig. 5B). We also found that rats with focal cerebral ischemia spent less time in the target quadrant compared to the sham rats. EA pretreatment significantly prolonged the time spent swimming in the target quadrant following MCAO (EA + MCAO vs. MCAO, P < 0.05; Fig. 5C). Consistent with this finding, behavioral tracking showed increased exploration time in the target quadrant for the EA + MCAO and EA + PBS + MCAO groups compared with the MCAO group. Dkk-1 inhibited the beneficial effects of EA pretreatment on learning and memory functions (Fig. 5D). No significant differences were detected between the MCAO and Dkk-1 + MCAO groups or between the EA + PBS + MCAO and EA + MCAO groups.

3.6. The Wnt/β-catenin agonist LiCl replicated the neuroprotective effects of EA pretreatment

Twenty-four hours after reperfusion, the Bcl-2/Bax ratio was evaluated in the LiCl and EA pretreatment groups and in the MCAO group. As shown in Fig. 6A, the Bcl-2/Bax ratio was more robust in the former two groups than it was in the latter group (LiCl + MCAO vs. MCAO, P < 0.05; EA + MCAO vs. MCAO, P < 0.05; Fig. 6A), while it was similar for the LiCl + MCAO and EA + MCAO groups. No significant differences in the Bcl-2/Bax ratio were identified between the MCAO group and the NS + MCAO group.

As shown in Fig. 6B, the β-catenin protein expression levels were significantly higher in the LiCl + MCAO and EA + MCAO groups than they were in the MCAO group (P < 0.05). The β-catenin expression levels were comparable between the LiCl + MCAO and EA + MCAO groups.

Fig. 2. Electroacupuncture (EA) pretreatment upregulates the expression of β-catenin after reperfusion. (A, B) Western blot analysis for β-catenin expression 24 h after reperfusion (n = 10). The β-catenin protein expression level is significantly lower in the middle cerebral artery occlusion (MCAO) group than it is in the control group. EA pretreatment significantly increases this expression. (C) Immunoreactivity of β-catenin in the hippocampus (n = 6–8). *P < 0.05 vs. the control group, †P < 0.05 vs. the MCAO group.

Fig. 3. Western blot analysis for the effects of electroacupuncture (EA) pretreatment and Dkk-1, a Wnt/β-catenin antagonist, on β-catenin expression (n = 10) 24 h after reperfusion. Pretreatment with EA significantly increases the expression of β-catenin 24 h after reperfusion. Dkk-1 (0.1 μg/mL) administered 30 min before ischemia reverses the effects of EA pretreatment. *P < 0.05 vs. the sham group, †P < 0.05 vs. the MCAO group, ‡P < 0.05 vs. the EA + MCAO group.

3.6. The Wnt/β-catenin agonist LiCl replicated the neuroprotective effects of EA pretreatment

Twenty-four hours after reperfusion, the Bcl-2/Bax ratio was evaluated in the LiCl and EA pretreatment groups and in the MCAO group. As shown in Fig. 6A, the Bcl-2/Bax ratio was more robust in the former two groups than it was in the latter group (LiCl + MCAO vs. MCAO, P < 0.05; EA + MCAO vs. MCAO, P < 0.05; Fig. 6A), while it was similar for the LiCl + MCAO and EA + MCAO groups. No significant differences in the Bcl-2/Bax ratio were identified between the MCAO group and the NS + MCAO group.

As shown in Fig. 6B, the β-catenin protein expression levels were significantly higher in the LiCl + MCAO and EA + MCAO groups than they were in the MCAO group (P < 0.05). The β-catenin expression levels were comparable between the LiCl + MCAO and EA + MCAO groups.
4. Discussion

A previous study revealed the neuroprotective effects of EA pretreatment on the ischemic penumbra, which manifested as significant inhibition of neuronal apoptosis, reduced infarct volume, and improved neurological deficits 24 h after reperfusion (Zhou et al., 2013). Compared to other neural regions, the hippocampus is discerningly vulnerable to I/R injury. Ischemic hippocampal pyramidal cell death in both rodents and humans is characterized by selective neuronal loss that typically occurs 24–72 h after reperfusion (Ghosh et al., 2013). Therefore, in the present study, we chose to focus on the neuroprotective effects of EA pretreatment on the hippocampus and aimed to identify the endogenous signaling pathway that underlies this process. Our results showed that EA pretreatment induced rapid tolerance to focal cerebral ischemia, as represented by increased neuronal survival, improvement of neurological outcomes, and restoration of learning and memory functions 24 h after reperfusion. However, Dkk-1, a Wnt/β-catenin antagonist, reversed the beneficial effects of EA pretreatment. These results suggest that the Wnt/β-catenin signaling pathway is involved in the mechanisms underlying EA pretreatment-induced neuroprotection.

Ischemic tolerance is defined as a transient resistance to lethal ischemia induced by a prior sub-lethal stimulus (i.e., preconditioning). Two types of ischemic tolerance in the brain have been documented in the literature: rapid and delayed (Durukan and Tatlisumak, 2010). Tolerance can be induced by a variety of stimuli, including repetitive hypoxic preconditioning, remote ischemic preconditioning, and pharmacological preconditioning (Vijayakumar et al., 2015; Wang et al., 2015). Recent studies have suggested that EA pretreatment at the Baihui acupoint (GV20) prior to lethal ischemic insult induces rapid tolerance (Zhou et al., 2013) and triggers endogenous protective responses involving the endocannabinoid system (Wang et al., 2009). Typically, pretreatment also influences the activity of adenosine A1 receptors (Wang et al., 2005) and related signaling elements, including mitogen-activated protein kinase kinases 1 and 2, extracellular signal-regulated kinases 1 and 2 (Du et al., 2010), and Notch signaling (Zhao et al., 2015). Interestingly, a recent study on AD demonstrated that Wnt/β-catenin may be potential mediators of the beneficial effects of acupuncture (Zhou et al., 2014). Wnt proteins, which are widely distributed throughout the brain, are extracellular factors that play key roles in the developing and mature central nervous system (Lambert et al., 2015). It has been shown that Wnt signaling regulates diverse cellular processes, including cell proliferation, cell polarity, and cell death. Following Wnt/β-catenin activation, cytoplasmic β-catenin becomes stabilized and enters the nucleus, where it associates with transcription factors, notably T cell factor and lymphoid enhancer factor, to regulate the transcription of target genes (Huang et al., 2015). Moreover, the Wnt/β-catenin signaling pathway reportedly induces blood–brain barrier properties in neural endothelial cells during brain angiogenesis and postnatal vascular maturation (Liebner et al., 2008). Activation of the Wnt signaling pathway also contributes to functional recovery and induces neuroprotective processes and neurogenesis after focal cerebral ischemia (Sun et al., 2014). One study showed that administration of the Wnt/β-catenin agonist LiCl elicited neuroprotective effects with regard to learning and memory after I/R injury (Fan et al., 2015). In contrast, Dkk-1, a Wnt/β-catenin antagonist, is required for and exacerbates ischemic and excitotoxic
**Fig. 5.** Effects of electroacupuncture (EA) pretreatment on cognitive function in the presence of Dkk-1, a Wnt/β-catenin antagonist, as assessed using the Morris water maze (MWM) test at 24 h after reperfusion (n = 12): (A) Changes in the escape latency (i.e., the time required to locate and climb onto the platform) during six-block acquisition trials. EA pretreatment decreases the escape latencies in all trials; this is reversed by Dkk-1. (B, C) Effects of EA pretreatment with or without Dkk-1 on (B) swimming speed and (C) time spent in the target quadrant. (D) The swimming tracks of rats in different conditions in the probe trial test. *P < 0.05 vs. the sham group, #P < 0.05 vs. the MCAO group, **P < 0.05 vs. the EA + MCAO group, ***P < 0.05 vs. the EA + MCAO group.

**Fig. 6.** Western blot analysis for the effects of lithium chloride (LiCl), a Wnt/β-catenin agonist, 24 h after reperfusion in rats with ischemia/reperfusion injury induced by 120 min of middle cerebral artery occlusion (MCAO; n = 10). (A) The expression of Bcl-2 and Bax in the hippocampus 24 h after reperfusion and relative changes in the Bcl-2/Bax ratio. (B) Representative western blots of β-catenin 24 h after reperfusion. *P < 0.05 vs. the sham group, #P < 0.05 vs. the MCAO group, **P < 0.05 vs. the MCAO group.
neuronal death (Mastroiacovo et al., 2009). Additionally, the inactivation of β-catenin by small interfering RNA increased the stroke-induced infarct volume in adult rats (Lei et al., 2012). Wnt/β-catenin also plays a critical role in the neuroprotective effects of preconditioning against lethal I/R injury in the heart (Vigneron et al., 2011), kidneys (Kunczewitch et al., 2015), and liver (Li et al., 2015). These findings suggest that Wnt/β-catenin regulation plays a major role upstream and downstream of ischemic stroke episodes. However, little is known about the role of Wnt/β-catenin in the neuroprotective processes induced by EA pretreatment prior to cerebral I/R injury.

To explore the mechanisms and roles of Wnt/β-catenin in EA pretreatment-induced neuroprotection, in the present study, the β-catenin levels were assessed in the hippocampus, and a significant increase in expression was identified in the EA pretreatment group 24 h after reperfusion. Moreover, immunohistochemistry data revealed a higher constitutive expression of this protein. These findings suggest that β-catenin participates in the neuroprotective effects induced by EA pretreatment in models of acute stroke.

In subsequent experiments, rats received an intracerebroventricular injection of the Wnt/β-catenin inhibitor Dkk-1 after EA pretreatment to determine whether Wnt/β-catenin activation is involved in the neuroprotective programs of ischemic stroke. To examine the direct effects of the drug on the outcomes of acute stroke, we examined the Bcl-2/Bax and β-catenin levels, considering the association between Wnt signaling and Bcl-2/Bax activity. Since Bcl-2/Bax activity is critical for the activation/deactivation of cellular apoptotic machinery, it was probable that these proteins would be involved in the processes underlying stroke pathogenesis (Scalli et al., 2006). The results revealed that Dkk-1 reversed the upregulation of β-catenin, reduced the Bcl-2/Bax ratio, and suppressed the rapid ischemic tolerance induced by EA pretreatment.

To determine whether EA pretreatment could also reverse the learning and memory deficits induced by cerebral ischemia, the MWM test was conducted 24 h after reperfusion. Our study demonstrated that EA stimulation significantly restored spatial learning and memory deficits after reperfusion; these effects were reversed by the intracerebral infusion of Dkk-1. These results suggest that EA stimulation might potentially be effective at ameliorating the cognitive impairments induced by cerebral ischemia, with the protective effects being mediated by Wnt/β-catenin. Such findings are supported by data from previous studies demonstrating that the activation of Wnt signaling modulated cognitive function in AD models (Vargas et al., 2014) and that EA stimulation significantly reversed the learning and memory deficits observed in mouse models of AD (Li et al., 2014).

Previous studies showed that LiCl, a Wnt/β-catenin agonist, is involved in the amelioration of the neurotoxic effects of ischemic injury in CA1 neurons (Caraci et al., 2008) and the decrease in focal ischemia-induced neurological abnormalities associated with Wnt signaling (Nonaka and Chuang, 1998). LiCl, which persists as the preferred course of treatment for patients with bipolar disorder, has a narrow therapeutic window. Subsequently, small alterations in serum concentration could result in serious adverse effects, wherein even concentrations within the therapeutic limits might induce toxicity. Previous in vivo studies of cerebral ischemia demonstrated that LiCl must be chronically administered to elicit neuroprotective effects (Nonaka and Chuang, 1998; Nonaka et al., 1998). However, LiCl administration does not produce an instant therapeutic effect; instead, there is a variable delay in the efficacy of LiCl that is not immediately reversed upon its discontinuation. Therefore, the mechanisms underlying the therapeutic activity of LiCl appear to involve long-term effects, perhaps by altering ion homeostasis or neurotransmitter receptor balance. Comparatively, Yoshida et al. (1991) demonstrated that a single injection of LiCl prior to ischemia onset failed to protect hippocampal neurons against transient ischemia in gerbils. In the present study, LiCl was administered for 7 days, as described in a previous experiment (Scalli et al., 2006), to assess whether administering this Wnt/β-catenin agonist could replicate the beneficial effects of EA pretreatment. The current study provides evidence to suggest that 7 days of LiCl injections significantly upregulated the expression level of β-catenin and decreased the Bcl-2/Bax ratio when administered prior to I/R injury.

5. Conclusions

In conclusion, the findings of the current study suggest that the Wnt/β-catenin upregulation induced by EA pretreatment plays a crucial role in neuroprotection, with the inhibition of Wnt/β-catenin resulting in the loss of neuronal protection. While further studies are necessary to elucidate the detailed sequence of reactions based on Wnt/β-catenin upregulation, the present findings further our understanding of the mechanisms underlying EA-induced tolerance to focal cerebral ischemia and support the theoreti
cal and scientific basis of this pretreatment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

Baarsma, H.A., Königshoff, M., Gosen, R., 2013. The WNT signaling pathway from ligand secretion to gene transcription: molecular mechanisms and pharmaco-
Caraci, F., Bucci, C., Biagioni, F., Aronica, E., Mastroiacovo, F., Cappuccio, L., et al., 2008. The Wnt antagonist, Dickkopf-1, as a target for the treatment of neurod
Cho, J.H., Yan, B.C., Lee, Y.J., Park, J.H., Ahn, J.H., Kim, J.H., et al., 2013. Reduced beta-
vation in electroacupuncture pretreatment via cannabinoid CB1 receptor in rats. Brain Res. 1360, 1–7.
Ghosli, A., Sarkar, S., Mandal, A.K., Das, N., 2013. Neuroprotective role of nano-
capsulated quercetin in combating ischemia-reperfusion induced neuronal damage in young and aged rats. PLoS One 8, e57735.


