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Lithium chloride promotes neural functional recovery after local cerebral ischaemia injury in rats through Wnt signalling pathway activation

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Background: *Lithium chloride (LiCl) has a significant neuroprotective effect in cerebral ischaemia. However, to date, there is a paucity of evidence on the role of LiCl in neural restoration after brain ischaemia and the signalling pathways involved remain unclear.*

Materials and methods: *Therefore, to address this gap, the middle cerebral artery occlusion (MCAO) rat model was used to simulate human ischaemia stroke. Male Sprague-Dawley rats were given MCAO for 90 min followed by reperfusion, and Dickkopf-1 (DKK1, 5.0 μg/kg) was administered half an hour before MCAO. Rats were then treated with hypodermic injection of LiCl (2.0 mmol/kg) twice a day for 1 week. After treatment, cognitive impairment was assessed by the Morris water maze test. Neurological deficit score, 2,3,5-triphenyl tetrazolium chloride staining, brain water content, and histopathology were used to evaluate brain damage. Enzyme-linked immunosorbent assay was used to measure oxidative stress damage and inflammatory cytokines. Apoptosis of the hippocampal neurons was tested by western blot. The key factors of Wnt signalling pathway in the ischaemic penumbra were detected by immunofluorescence staining and quantitative real-time polymerase chain reaction.*

Results: *Current experimental results showed that LiCl treatment significantly improved the impaired spatial learning and memory ability, suppressed oxidative stress, inflammatory reaction, and neuron apoptosis accompanied by attenuating neuronal damage, which subsequently decreased the brain oedema, infarct volume and neurological deficit. Furthermore, the treatment of LiCl activated Wnt signalling pathway. Interestingly, the aforementioned effects of LiCl treatment were markedly reversed by administration of DKK1, an inhibitor of Wnt signalling pathway.*

Conclusions: *These results indicate that LiCl exhibits neuroprotective effects in focal cerebral ischaemia by Wnt signalling pathway activation, and it might have latent clinical application for the prevention and treatment of ischaemic stroke.*
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Key words: lithium chloride, cerebral ischaemia, Wnt signalling pathway, neuroprotection, rats

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INTRODUCTION

Stroke is a leading cause of disability and death in the United States and Great Britain, affecting 0.2% of the population each year [18]. It is reported that there are about 2.0 million stroke patients in China every year [8]. Strokes were classified as ischaemic and haemorrhagic, more than 75% of cases being ischaemic [25]. Although there have been significant clinical improvements in the treatment of ischaemic stroke, many patients remain severely disabled, effectiveness of treatment is finite [35]. Therefore, the search for potential new drug intervention targets is critical for treatment to reduce stroke-related brain injury.

At present, lithium chloride (LiCl; molecular weight 42.39) is widely used in the clinical treatment of affective disorders [31]. LiCl has recently been reported to play an unexpected neuroprotective role in animal models of various neurodegenerative diseases [5, 12]. For example, LiCl has been shown to activate the glycogen synthase kinase 3β (GSK-3β) related pathway and participate in the pathophysiological process of Alzheimer's disease; the overactivation and overexpression of GSK-3β lead to amyloid-β-induced neurotoxic damage, which can be normalised by lithium ion [6]. In a rat model of cerebral ischaemia, chronic LiCl treatment significantly reduced cerebral infarction volume and neurological deficits caused by permanent middle cerebral artery occlusion (MCAO) [13]. In addition, the neuroprotection of LiCl is associated with several signal transduction pathways in the nerve functional restoration after ischaemic stroke [11, 38]. Among these, the Wnt signalling pathway is associated with neuronal differentiation, development, and migration [1, 37]; the activation of Wnt signalling pathway can be induced by abnormal changes of key molecules in Wnt-3a, dishevelled-1 (Dvl-1), axis inhibition protein (Axin) and β-catenin [3]. However, few studies have investigated the neuroprotective mechanisms induced by LiCl post-conditioning via the Wnt signalling pathway. Based on these findings, we employed a rat model with MCAO to confirm the hypothesis that LiCl promotes neuroprotective effects after ischaemic stroke by activating the Wnt signalling pathway.

MATERIALS AND METHODS

Animals and ethics

For this study, 100 adult male Sprague-Dawley rats (4–5 months old, weighing 330–350 g) were purchased from the laboratory animal centre of Guizhou Medical University, P.R. China (clean grade, License No. SCXK [qian] 2018-0001). Rats were housed in a cage at humidity (50–70%), temperature (22–24°C) and a 12 h light/dark cycle. Moreover, water and food were supplied ad libitum during the period of the experiment. The study protocol was approved by the Experimental Animal Research Committee of Guizhou Medical University of China on March 3, 2021 (approval No. 2100034). During the study, all efforts were made to reduce animal suffering and reduce the number of rats. Furthermore, all animal protocols were conducted under the guidelines of the Ministry of Science and Technology of the People's Republic of China ([2006]398).

Animal model and experimental grouping

All rats were fed for 7 days before surgery to acclimatise to the environment. In this experiment, MCAO rat model was established according to slightly modified Zea-Longa's method [17]. Briefly, rats were anesthetised with pentobarbital sodium (50 mg/kg) and the skin of the neck was prepared and disinfected routinely. The right common carotid artery, external carotid artery, and internal carotid artery were exposed through a longitudinal incision of about 20 mm in the neck of rats, and the external carotid artery was ligated above the occipital artery. A longitudinal incision was made about 5.0 mm from the common carotid artery end. Nylon sutures with a diameter of 0.27 ± 0.03 mm were inserted along the internal carotid artery incision until slight resistance was felt, and the insertion depth was about 19.5 ± 0.5 mm. The artery was ligated at the distal part, clean the wound and suture the muscle and skin, disinfect the wound, and use local antibiotics to prevent infection. After 90 min of focal cerebral ischaemia, the nylon suture was withdrawn to form an ischaemia reperfusion injury. Sham-operated rats underwent the same procedure without the nylon suture. After surgery, which were maintained, the rats' body temperature of 37°C until recovery from anaesthesia. According to the principle of random block, rats were randomly divided into five groups (n = 20 for each group): sham operation (S) group, MCAO (M) group, MCAO + lithium (M+L) group, MCAO + Dickkopf-1 (DKK1) (M+D) group, and MCAO + lithium + DKK1 (M+L+D) group.

Drug administration

Lithium chloride (LiCl, Sigma-Aldrich, USA) was dissolved in 0.9% NaCl, and both the M+L group and

M+L+D group rats were then treated with LiCl twice a day at the dose of 2.0 mmol/kg for one consecutive week after MCAO operation according to formerly described [34]. The S group and M group rats simultaneously received equivalent volumes of normal saline injected. Both the M+D group and M+L+D group' rats were injected into the lateral ventricle of Dickkopf-1 (DKK1, 5.0 μg/kg, Rat DKK1 recombinant protein from R&D System from Minnesota of USA) before 30 min according to the previous description by He et al. [9]. In brief, the rats were anaesthetised with pentobarbital sodium by intraperitoneal injection, and then fixed into a brain stereotaxic instrument. According to the stereotactic brain atlas [21], longitudinal incision was made along the middle line of the parietal bone of rats, then haemostasis, periosteum incision was performed to expose the skull, and the right ventricle of rats was located (0.8 mm posterior to the bregma, 1.5 mm lateral to the midline, and 4.5 mm ventral from the skull surface), and a small hole with a diameter of 1.0 mm was drilled into the skull with a dental drill. A total of 2.5 μL (5.0 μg/kg) of DKK1 solution was subsequently injected over 5 min through a microsyringe into the rat right lateral ventricle. The needle was left in place for 5 min after the injection, and then removed, penicillin powder was sprayed over the incision, and the skin was sutured.

Cognitive function assessment

Cognitive deficits were tested by the Morris water maze (MWM) test based on formerly described [24] with modifications. The water maze apparatus (from Anhui Zhenghua Biological Instrument Equipment Co., LTD, China) consisted of a cylindrical stainless steel water tank with a diameter of 150.0 cm × a height of 60.0 cm, and filled with 22.5 ± 0.5°C. The water was becoming opaque through adding black ink. A platform with a diameter of 10.0 cm was submerged 2.0 cm below the water surface and placed at the midpoint of one quadrant. The general testing process has been described in detail elsewhere. Animals were placed into the tank, facing the wall of the pool, and were allowed to circumnavigate the pool in search of the platform for four trials (90 s per trial) at 9:00 am each day every day from days 1 to 5 after the LiCl treatment. Escape latency (second) was recorded using an on-line image video tracking system to indicate the learning results. After the last trial, the platform was removed from the pool and each rat received one 90 s swim probe trial. The number of crossing the platform and swimming time in the quadrant of the platform were recorded to indicate the memory results. A video camera was installed above the pool centre; all behaviours were recorded for following analyses. Data was automatically performed using the MWM image process system (Chengdu Tai Meng Technology, China).

Measurements of neurobehavioral scores, brain water content, and infarct volume

According to Longa scoring method [17], neurobehavioral scores were performed on rats after treatment. The scoring criteria of Longa scoring method: 0 points, no symptoms of neurological impairment; 1 point, one side of the forepaw cannot completely extend, slight neurological function defect; 2 points, one side of the forelimb fully flexed and turned in a circle, with moderate neurological impairment; at 3 points, the body of the rats was tilted toward the paralyzed side, with severe neurological impairment; 4 points, unable to walk spontaneously, conscious loss. After modelling, rats with neurobehavioural scores of 0, 4 and death were excluded, and the rats with neurobehavioural scores of 1–3 were judged as successful modelling.

Brain water content (BWC) was tested by the standard wet-dry ratio method as previously described [29]. Briefly, rats were sacrificed and then brain was quickly taken out and positioned on a dry aluminium foil. Then, wet weight was confirmed by immediately weighing the two hemisphere slices. Dry weight was obtained after dehydrating the samples for one day at 100°C. BWC was calculated as a percentage according to the following formula: (wet weight − dry weight) / wet weight × 100%.

After behavioural ability, rats from per group were optional, selected, and euthanised. The brain was immediately taken out and frozen at –20°C for 30 min, then the olfactory bulb was removed, and the coronal surface was evenly sliced every 2.0 mm, the brain slices were put into 2.0% 2,3,5-triphenyl-tetrazolium chloride (TTC, Boster, China) for half-hour at 37°C in the dark, and fixed with 4.0% paraformaldehyde (Leagene Biotechnology, China) overnight. In the result, normal brain tissue stained red, while the infarct area appears a pale grey colour. TTC-stained sections were taken a picture, and image processing software (Version 2.0.1, Bethesda, USA) was used to analyse the infarct size. The infarct percentage of normal volume was calculated as: infarct size (%) =

= (contralateral area – ipsilateral non-infarct area) / / contralateral area × 100%.

Enzyme-linked immunosorbent assay (ELISA)

After LiCl treatment, the rats were sacrificed and the hippocampus was quickly isolated from the brain. Half of the tissues were tested for oxidative stress, and the other half of the tissues were tested by ELISA and western blot. In brief, the hippocampus was cut into many pieces and then grinded in a homogenisation buffer at 4°C. According to the operating instruction (Boster, China), some brain tissue homogenates were used to detect the levels of oxidative stress markers, including superoxide dismutase (SOD), malondialdehyde (MDA), reactive oxygen species (ROS) and glutathione peroxidase (GSH-Px), to evaluate the degree of oxidative stress injury; the other part was used to detect changes in inflammatory response related factors including tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) by ELISA.

Pathological evaluation

Rats were anaesthetised by pentobarbital sodium, and perfused via the aorta with 0.1 M phosphate buffered saline (PBS, pH 7.4) for 10 min, followed by fixation by 4.0% paraformaldehyde (PBS, pH 7.4) for 10 min. The brain was quickly taken out and postfixed in 4.0% paraformaldehyde for 4 h, and then dehydrated all night through gradient sucrose solutions (10%, 20%, 30%, and 50%) until completely submerged. The dehydrated brain tissues were embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek, Japan) under freezing conditions. Coronal sections with thickness of 8.0 μm were cut using a cryostat, and unbiased cell estimation was performed in the hippocampal CA1 region every 6 sections according to a systematic random sampling procedure. About 120~150 continuous sections were collected from hippocampus in every rat and used to Nissl staining and immunofluorescence.

Nissl staining

Slice was putted in 0.02% toluidine blue (Beyotime Biotechnology, China) for 15 min at indoor temperature, dehydrated twice by a graded ethyl alcohol (50%, 70%, 90%, 95%, and 100%), permeabilised with xylene, wet-placed onto glass slides, and quickly mounted using neutral resin. Nissl positive neuron from five slices of each rat was counted with a light microscope (Olympus, Japan). The mean value of Nissl positive neuron for every rat was gained as the number of neuron in the rats.

Immunofluorescent staining

In order to investigate the regulatory role of Wnt signalling pathway, β-catenin or Wnt-3a positive cell in the ischaemic hippocampus was detected by immunofluorescence staining. Brain section was incubated for half-hour in 2.0 M HCl to denature DNA, and the reaction was neutralised in 0.1 M boric acid for 15 min. Thereafter, brain section was rinsed in PBS containing 0.3% Triton for half an hour, preincubated in 10% normal goat serum for 2 h at indoor temperature, and incubated with monoclonal rabbit anti-Wnt-3a or anti-β-catenin (1:150; Boster Biotechnology, Wuhan, China) at 4°C overnight, and then incubated with Cy3-conjugated affinity purified goat anti-rabbit IgG (1:100; Sigma, St. Louis, MO, USA) in a humidified chamber for 1 h at 37°C. Anti-Wnt-3a or anti-β-catenin was used as cell-type specific markers in each brain section. The number of β-catenin or Wnt-3a positive cells was analysed by laser scanning confocal microscope analysis system (Olympus, Japan).

Western blot assay

Western blot analysis was performed on hippocampal tissue from the ischaemic hemisphere [4]. In brief, 100 mg brain tissue samples from the ischaemic hippocampus were isolated and homogenised in a radio immunoprecipitation analysis lysis buffer (Sigma, USA) containing a protease inhibitor. The protein concentration was measured by the bicinchoninic acid method (Sigma, USA). Proteins were isolated by 10% SDS-PAGE gel electrophoresis and then transferred to PVDF membranes. It was blocked in 5.0% skim milk and incubated with a primary antibody of Bcl-2 or Bax protein (1:100 dilution; Beyotime Biotechnology, China) one night at 4°C, then incubated with the peroxidase-conjugated rabbit anti-goat secondary antibodies IgG (1:200 dilution; Sigma) for 2 h at room temperature. This was probed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for loading control. Immunoreactive protein was visualised by enhanced chemiluminescence and a western blotting detection system (BlotCycler, USA).

Quantitative polymerase chain reaction (q-PCR)

According to the manufacturer's instructions, Total RNA was extracted from rat hippocampal CA1 area with the TRIzol reagent (sigma, USA). And the

concentration of RNA was measured by an ultraviolet visible spectrophotometer (Beckman, USA). Vertibialiti® 96-Well Thermal Cycler instrument and cDNA Synthesis Reagents Kit (Super-Script™ III First-Strand Synthesis Super-Mix for q-PCR, USA) were applied to reverse transcription. Finally, the relative expression levels of Wnt-3a, Dvl-1, β-catenin, Axin-2, and GSK-3β were detected using a fluorescence quantitative PCR apparatus (Eco 48, Colpalmer Instruments, Shanghai, Co., LTD) and analysed by $2^{-\triangle\triangle Ct}$ method [28] and normalisation to GAPDH. Primer sequences used in this study were listed as follows: β-catenin (forward primer: 5′-GGGCGGCACCTTCCTACTTC-3′, reverse primer: 5′-GACCTGGAAAACGCCATCAC-3′), Wnt-3a (forward primer: 5′-GACTATCCGGCAGTTGCGAAGT-3′, reverse primer: 5′-CCACCCAGCCAC GAGACTCT-3′), Axin-2 (forward primer: 5′-CCTTGCCAAAA CGGAATACGA AAGG-3′, reverse primer: 5′-GG ACTTGCTCTGACGCTCACTCT-3′), GSK-3β (forward primer: 5′-CCACCATCCTTATCCCTCCTCA -3′, reverse primer: 5′-CGTTATTGGTCTGTCCACGGTCT -3′), and Dvl-1 (forward primer: 5′-CCTTCC ATCCAAATGTTGCCAGTA-3′, reverse primer: 5′-GGGCAGC CTCATCACGGTTT-3′).

Statistical analysis

The results represent the means ± standard errors and analysed by SPSS 20.0 software (SPSS Inc., Chicago, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey test. Data with equal variance were compared intergroup by the least significant difference method; while data with unequal variances were compared between groups by Tamhane's T2 method. $P < 0.05$ was considered statistically significant. $P < 0.05$ was considered to indicate a statistically difference.

RESULTS

LiCl improved the cognitive deficits in rats with cerebral ischaemia-induced injury

Cognitive function was assessed with MWM test after LiCl treatment (Fig. 1). There is an evident increase in escape latency in rat's navigation test in the M group ($F_{group} = 48.28$, $p < 0.05$). In contrast, LiCl treatment clearly reduced the navigation test's escape latency ($F_{group} = 51.37$, $p < 0.05$, Fig. 1A). On the third day, the rat's path in the M+D group is complex, while rats in the M+L group find the platform easily (Fig. 1A, C). The swimming path of the rat of each group on the fifth day is shown in Figure 1D. As shown in Figures 1B, 1C rats in the M+L group exhibited a shorter time in the target quadrant and a lower frequency of crossing the probe test platform than both the M group and the M+D group ($F_{group} = 72.34$, $p < 0.05$). These data collectively indicated that LiCl exerted beneficial effects on learning and memory.

LiCl promotes the recovery of neurological function in rats with cerebral ischaemia-induced injury

Neurological deficits were evaluated after LiCl treatment. The test analysis results are shown in Figure 2A. Because no neurological impairment occurred in the S group, the average neurological deficit score was 0, but the M group rats showed serious neurological deficit scores compared with the S group ($F_{group} = 22.35$, $p < 0.05$). Compared with the M group, the average neurological deficit scores were significantly reduced in both the M+L and the M+L+D groups, particularly in the M+L group ($F_{group} = 65.37$, $p < 0.05$). When pretreatment with the Wnt inhibitor DKK1 was performed, the neurological deficit scores of both the M+D group and the M+L+D group were clearly increased compared with those of both the S group and the M+L group ($F_{group} = 47.28$, $p < 0.05$), respectively. These results indicate that LiCl treatment clearly improved neurological impairment; nevertheless, the Wnt signalling inhibitor DKK1 restrained this neuroprotective effect.

LiCl attenuated cerebral oedema in rats with cerebral ischaemia-induced injury

Brain water content was used to assess brain oedema following LiCl treatment, as shown in Figure 2B. Compared with the S group, the BWC was clearly increased in the M group ($F_{group} = 22.49$, $p < 0.05$). After LiCl treatment, compared with the M group, the BWC was significantly decreased in both the M+L group and the M+L+D group ($F_{group} = 76.53$, $p < 0.05$), particularly in the M+L group ($F_{group} = 85.26$, $p < 0.05$). In addition, when the Wnt signal pathway inhibitor DKK1 was injected intracerebroventricularly, there were obvious differences in both the M+D group ($F_{group} = 47.36$, $p < 0.05$) and the M+L+D group ($F_{group} = 42.16$, $p < 0.05$). However, there were no significant changes in the contralateral hemisphere.

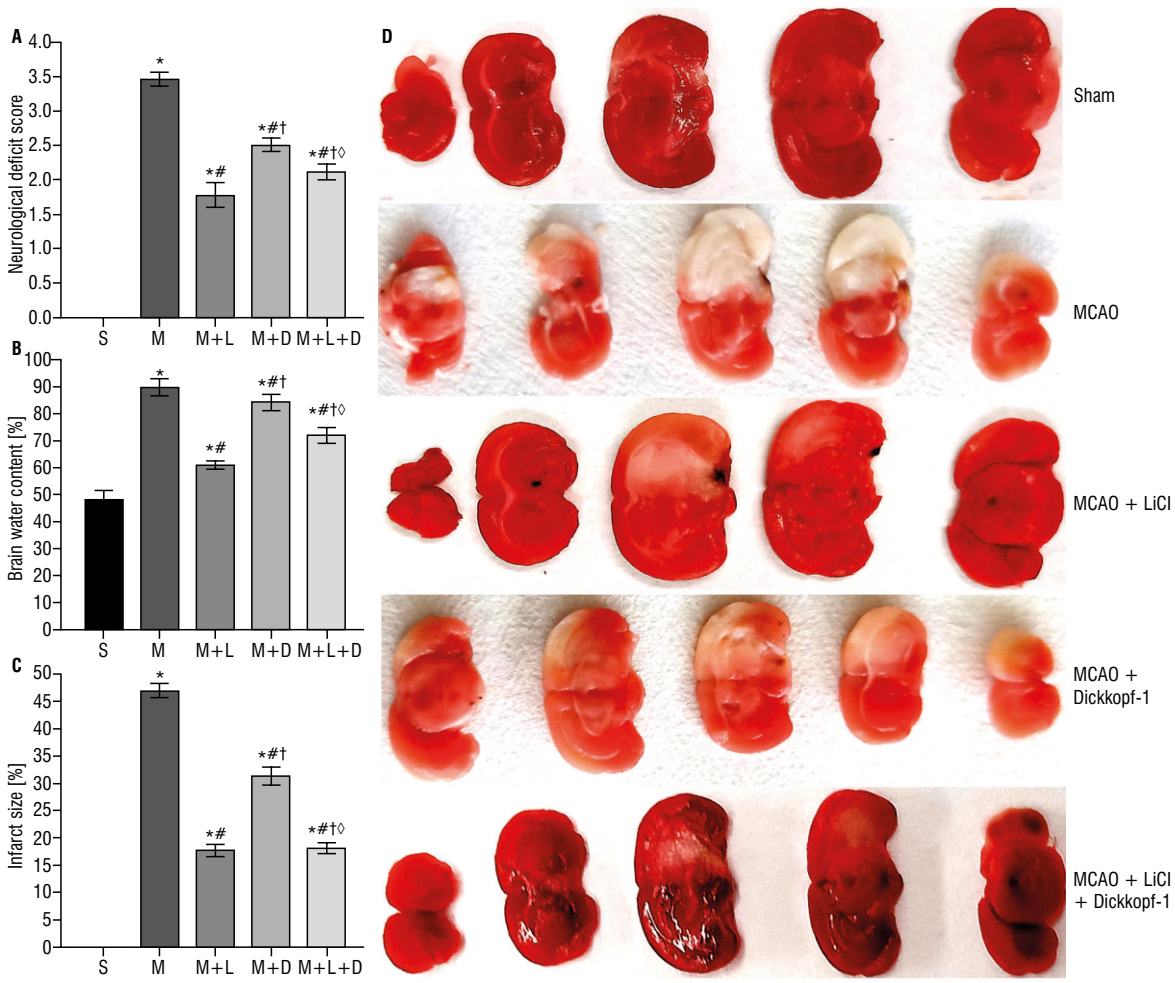

Figure 1. Effects of lithium chloride (LiCl) treatment on neurobehavioral ability in rats. Escape latency (**A**) and pathway (**D**) during platform trials, time spent in the target quadrant (**B**), and the number of crossings through the platform (**C**) in the probe test; S — sham; M — middle cerebral artery occlusion; L — lithium chloride; D — Dickkopf-1. The results are expressed as the mean ± standard error of 20 animals in each group and analysed by one-way ANOVA, followed by the Student–Newman–Keuls test; *$p < 0.05$ vs. S group; #$p < 0.05$ vs. M group; †$p < 0.05$ vs. M+L group; ◊$p < 0.05$ vs. M+D group; MCAO — middle cerebral artery occlusion.

LiCl reduced the infarct size in rats with cerebral ischaemia-induced injury

The area of cerebral infarction in MCAO-induced rats was determined by TTC staining (Fig. 2D). No infarct tissue was observed in the S group, and the infarct area (white) was very obvious in the M group. Extensive infarcts in the cortical and subcortical areas were observed in a series of brain sections, accounting for about half of the total infarcts in the cortical and subcortical areas (Fig. 2D). Compared with the M group and the M+D group, the infarct area of LiCl treated rats was significantly reduced ($F_{group} = 78.26$, $p < 0.05$). However, there were no significant differences in both the M+L group and M+L+D group after the Wnt signalling pathway suppressor DKK1 was administered ($F_{group} = 126.67$, $p > 0.05$, Fig. 2C).

LiCl relieved the oxidative stress damage in rats with cerebral ischaemia-induced injury

To evaluate the effects of LiCl on oxidative stress injury, the levels of MDA, SOD, GSH-Px, and ROS were measured (Fig. 3A, B). SOD and GSH-Px expression were significantly decreased in the M group compared with the S group ($F_{group} = 37.25$, $p < 0.05$; Fig. 3A, B). Compared to the S group, MAD and ROS expression were significantly increased in the M group ($F_{group} = 41.59$, $p < 0.05$; Fig. 3A, B). LiCl treatment resulted in a significant increase in the levels of MDA and ROS, and a remarkable decrease in the levels of SOD and GSH-Px in the brain tissues of rats, indicating a rise in oxidative stress in the rats with cerebral ischaemia. On the other hand, the Wnt signalling pathway inhibitor DKK1 attenuated LiCl antioxidant activity.

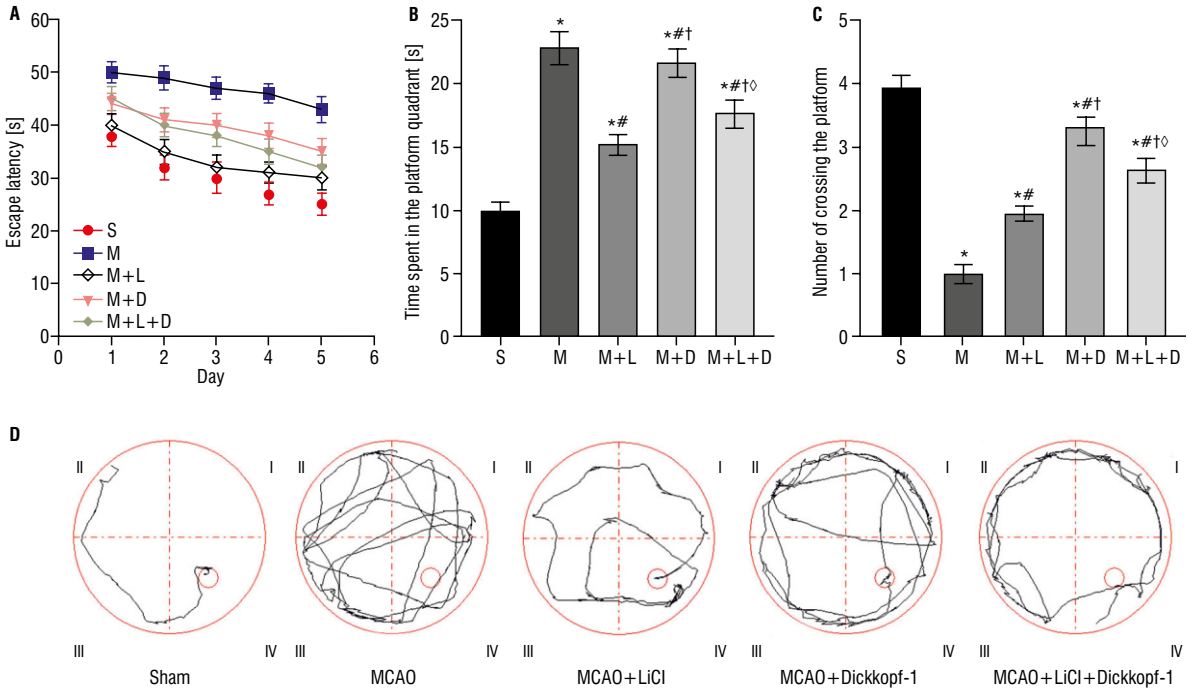

Figure 2. Neurological deficit scores of cerebral middle cerebral artery occlusion (MCAO) rats following lithium chloride (LiCl) treatment in the five groups (**A**, n = 20). Effect of lithium chloride treatment on brain water content (**B**, n = 5). Administration of lithium chloride reduced the infarct size (**C**, n = 5), Infarct size of the ischaemic cortex was measured, normalised to the contralateral cortex, and expressed as a percentage according to the following formula: Infarct size (%) = (contralateral area – ipsilateral non-infarct area) / contralateral area × 100%; **D.** Representative cerebral infarcts stained by a 2.0% 2,3,5-triphenyl tetrazolium chloride (TTC) solution. Normal brain tissue stained red by TTC staining; the infarct area appears a pale grey colour; S — sham; M — middle cerebral artery occlusion; L — lithium chloride; D — Dickkopf-1. The results are expressed as the mean ± standard error of those animals in each group and were analysed by one-way ANOVA, followed by the Student–Newman–Keuls test; *$p < 0.05$ vs. S group; $^{\#}p < 0.05$ vs. M group; $^{\dagger}p < 0.05$ vs. M+L group; $^{\diamond}p < 0.05$ vs. M+D group.

LiCl suppressed the inflammatory response in rats with cerebral ischaemia-induced injury

To investigate the anti-inflammatory effects of LiCl in cerebral ischaemia-induced rats, the TNF-α and IL-6 concentrations in the hippocampal CA1 region were investigated after the rats were sacrificed. The concentrations of TNF-α and IL-6 were significantly increased in the M group. However, after LiCl treatment, the proinflammatory factors TNF-α and IL-6 were significantly reduced ($F_{group} = 51.68$, $p < 0.05$; Fig. 3C). The Wnt signalling pathway inhibitor DKK1 reversed this protective effect.

LiCl against apoptosis in rats with cerebral ischaemia-induced injury

To further clarify the molecular mechanisms underlying the neuroprotective effect of LiCl, the analysis focused on two proteins involved in apoptotic death, Bcl-2 and Bax. Western blot assay revealed that after MCAO injury, Bcl-2 expression was significantly decreased in the M group compared to the S group ($F_{group} = 74.23$, $p < 0.05$; Fig. 3D, E). Treatment with LiCl significantly suppressed this reduction in the expression level. In addition, Bax expression was significantly increased in the M group compared to the S group, and this was significantly prevented by treatment with LiCl ($F_{group} = 67.86$, $p < 0.05$; Fig. 3D, E). However, the Wnt signalling pathway inhibitor DKK1 only reversed the effects on Bcl-2 expression.

LiCl relieved the histopathological injury in rats with cerebral ischaemia-induced injury

Nissl staining was applied to measure the infarct volume after MCAO. Because Nissl bodies are unique structures in neurons [30], Nissl staining can be used to specifically stain the Nissl bodies for neuropathological evaluation. Nissl staining of the hippocampal CA1 area showed that there were four or five layers of pyramidal cells in the sham group (Fig. 4A). These neurons were arranged regularly and compactly with no obvious fracture of the Nissl body. After cerebral MCAO, the arrangement of neurons in the hippocampal CA1 region was disordered compared to the S group. The boundary was dim, and the cell band

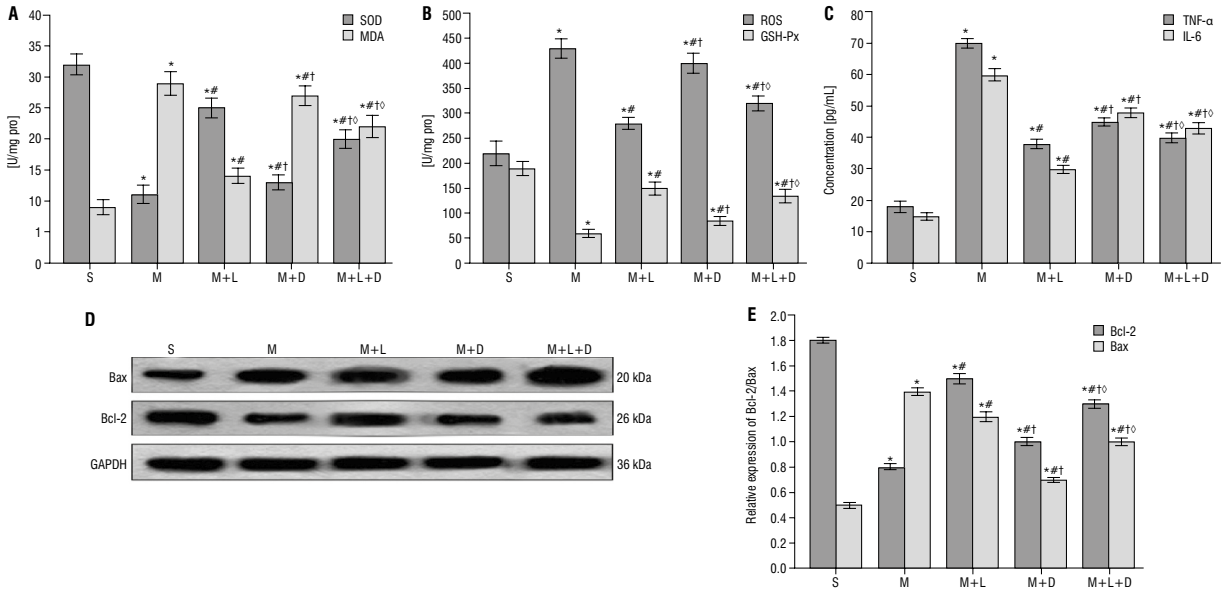

Figure 3. Effect of lithium chloride on oxidative stress damage, inflammatory cytokines, and apoptosis. The concentrations of malondialdehyde (MDA) and superoxide dismutase (SOD) (**A**), reactive oxygen species (ROS) and glutathione peroxidase (GSH-Px) (**B**), and interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) (**C**) in the cerebral tissues of treated rats were detected by ELISA. Bax and Bcl-2 protein expression in the hippocampus after cerebral ischaemia as analysed by western blot assay; **D.** Representative protein bands of Bax, Bcl-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown; **E.** Representative relative optical densities of protein bands from the ischaemic penumbra are shown in the bar graphs. Expression was calculated as the ratio of the optical density of the target protein to that of GAPDH; S — sham; M — middle cerebral artery occlusion; L — lithium chloride; D — Dickkopf-1. The results are expressed as the mean ± standard error of 10 animals in each group and were analysed by one-way ANOVA followed by the Student–Newman–Keuls test; *$p < 0.05$ vs. S group; $^{\#}p < 0.05$ vs. M group; $^{\dagger}p < 0.05$ vs. M+L group; $^{\diamond}p < 0.05$ vs. M+D group.

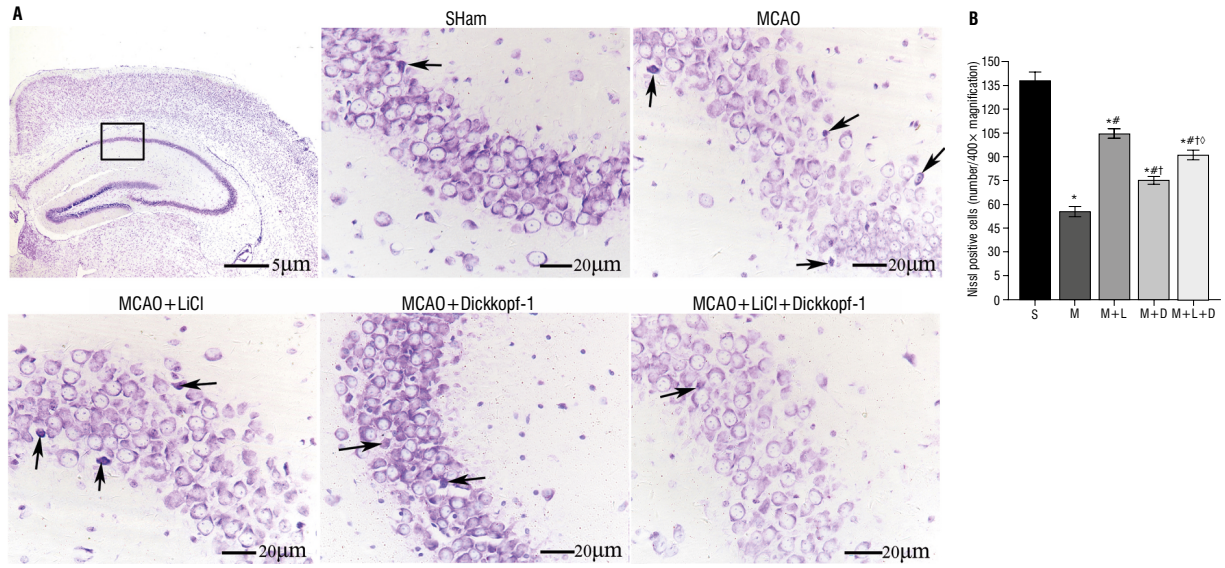

Figure 4. Representative brain sections of Nissl (cresyl violet) staining after brain ischaemia; **A.** Neurons in the hippocampal CA1 area. The box indicates the analysed region (Nissl staining, scale bar = 5.0 μm). In the sham group, the neurons were arranged regularly and the Nissl bodies in the cytoplasm were enriched; after cerebral middle cerebral artery occlusion (MCAO), the number of neurons was substantially reduced, and they were arranged in a disorderly manner. There was a reduction in the number of Nissl bodies in the cytoplasm (arrows indicate shrunken, darkened, damaged neurons). In rats given lithium chloride (LiCl) treatment, the number of neurons was increased and there were a greater number of Nissl bodies in the cytoplasm. When the Wnt signal pathway inhibitor Dickkopf-1 was injected intracerebroventricularly, many pyramidal cells were reduced in size and there were nuclear pyknosis and hyperchromatic nuclei. Scale bars = 20 μm; **B.** Quantitation of Nissl-positive cells; S — sham; M — middle cerebral artery occlusion; L — lithium chloride; D — Dickkopf-1. The results are expressed as the mean ± standard error of 5 animals in each group and were analysed by one-way ANOVA followed by the Student–Newman–Keuls test; *$p < 0.05$ vs. S group; $^{\#}p < 0.05$ vs. M group; $^{\dagger}p < 0.05$ vs. M+L group; $^{\diamond}p < 0.05$ vs. M+D group.

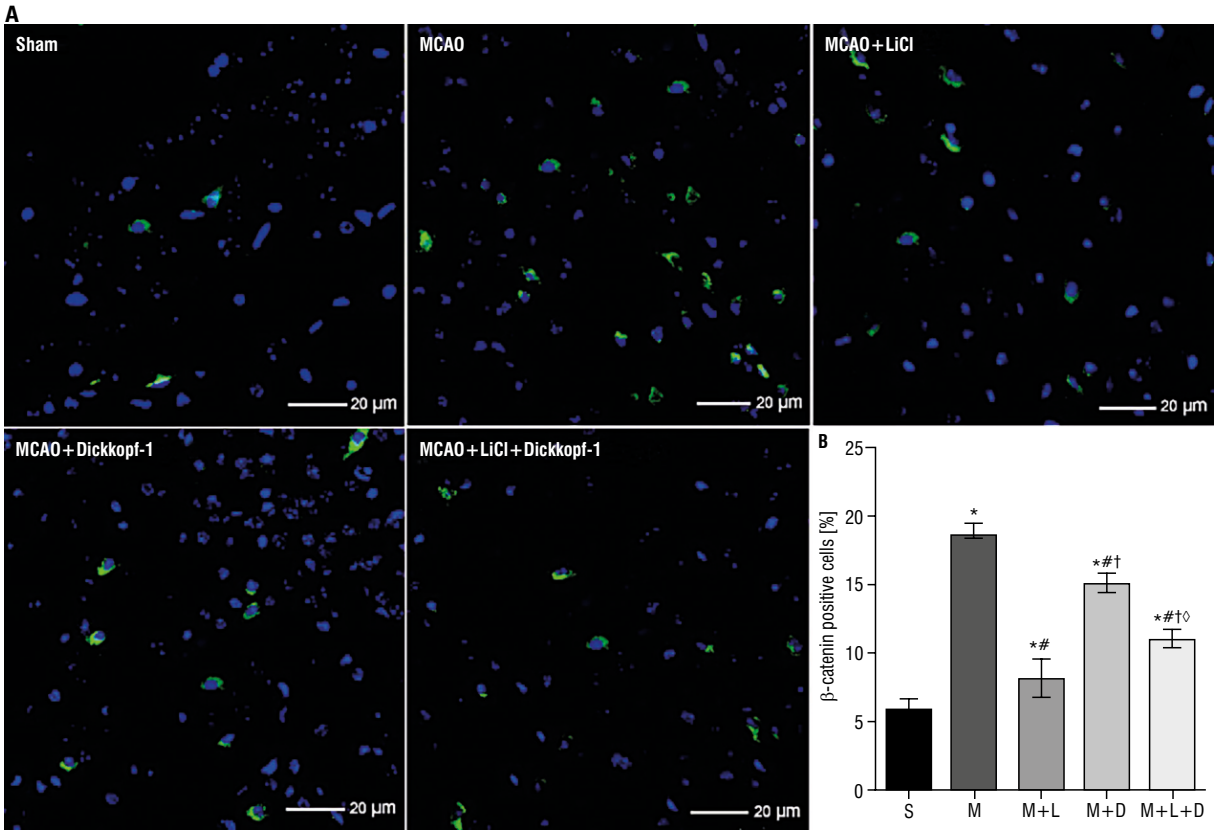

Figure 5. Effect of lithium chloride (LiCl) on β-catenin positive cells in rat parietal cortex; **A.** β-catenin positive cells were examined by immunofluorescence. β-catenin positive cells are labelled; these were mainly localized to the cytoplasm. Scale bars: 20 μm; **B.** Quantitation of β-catenin positive cells; S — Sham; M — middle cerebral artery occlusion; L — lithium chloride; D — Dickkopf-1. The results are expressed as the mean ± standard error of 5 animals in each group and were analysed by one-way ANOVA followed by the Student–Newman–Keuls test; *$p < 0.05$ vs. S group; #$p < 0.05$ vs. M group; †$p < 0.05$ vs. M+L group; ◊$p < 0.05$ vs. M+D group; MCAO — middle cerebral artery occlusion.

was irregular. There was a large reduction in the number of surviving cells and the Nissl body of many neurons was fragmented ($F_{group} = 85.52$, $p < 0.05$; Fig. 4A, B). In addition, these pathological changes in the hippocampal CA1 area of the M+L group were evidently reduced compared with the MCAO group; the M+L group exhibited almost normal morphology and structure of the pyramidal cells ($F_{group} = 57.46$, $p < 0.05$; Fig. 4A, B). Yet many pyramidal cells were reduced in size, with nuclear pyknosis and hyperchromatic nuclei in the M+L+D group ($p < 0.05$; Fig. 4A, B).

LiCl reduced β-catenin positive cells in rats with cerebral ischaemia-induced injury

Beta-catenin positive cells were examined by immunofluorescence. β-catenin protein immunofluorescent staining revealed intense green staining in the cytoplasm of neurons, particularly in the injured areas. The expression of the β-catenin protein in the rat parietal cortex is shown and quantified in Figure 5A, B. Compared with the small amount of expression in the S group, the expression of β-catenin positive cells was clearly increased in the M group ($F_{group} = 54.43$, $p < 0.05$). Treatment with LiCl significantly reduced the number of β-catenin positive cells compared to the M group ($F_{group} = 60.27$, $p < 0.05$). However, there was an obvious increase in the number of β-catenin positive cells in the M+L+D group compared to the M+L group ($F_{group} = 64.37$, $p < 0.05$).

LiCl activated the Wnt signalling pathway in rats with cerebral ischaemia-induced injury

To test the hypothesis that LiCl activates Wnt/β-catenin signalling, key proteins in the Wnt signalling pathway were evaluated by immunofluorescence and q-PCR after LiCl treatment, including Wnt-3a, Dvl-1, GSK-3β, β-catenin, and Axin-2. The expression levels of these indices reflect the activation of the relevant signalling pathway. The results indicated

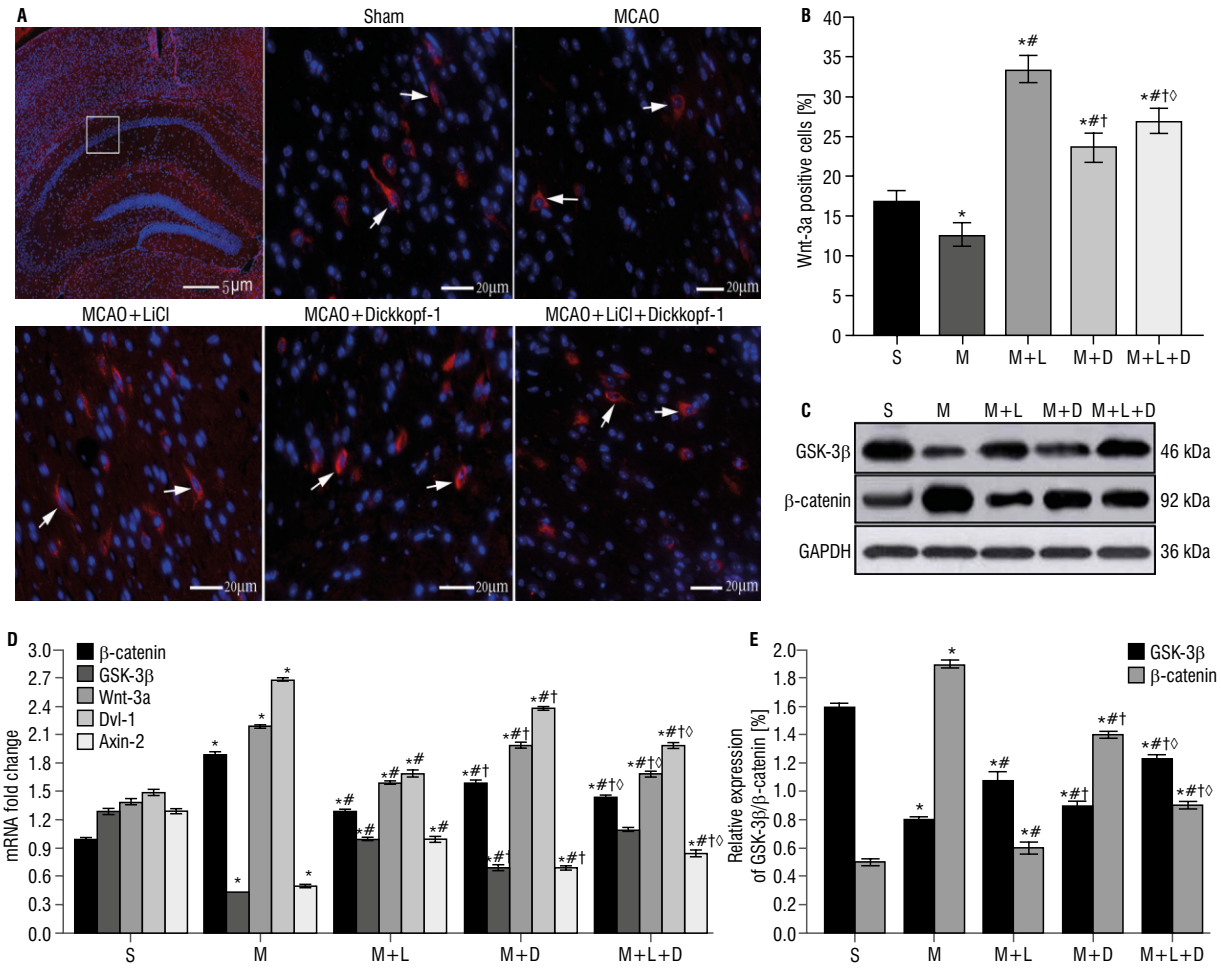

Figure 6. The key factors in the Wnt signalling pathway were examined by immunofluorescence staining and quantitative real-time polymerase chain reaction; **A.** Wnt-3a-positive cells are labelled; these were mainly localized in the cytoplasm. The red fluorescence indicates Wnt-3a and blue fluorescence indicates nuclei. The box indicates the analysed region (scale bars = 5.0 μm). Little positive staining was detected in the sham animals (arrows indicating Wnt-3a-positive neurons). Lithium chloride (LiCl) treatment promoted Wnt-3a-positive staining in the ischaemic penumbra; Wnt-3a-positive neurons were abundant in the CA1 region compared with the middle cerebral artery occlusion (MCAO) group. Scale bars = 20 μm; **B.** Quantitation of Wnt-3a-positive cells in the ischaemic penumbra is shown in the bar graph; **C.** Representative protein bands of glycogen synthase kinase 3β (GSK-3β), β-catenin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown, as determined by western blot assay. Expression was calculated as the ratio of the optical density of the target protein to that of GAPDH; **D.** Effects of lithium chloride on mRNA levels of Wnt-3a, β-catenin, dishevelled-1 (Dvl-1), GSK-3β, and axis inhibition protein 2 (Axin-2); **E.** Representative relative optical densities of protein bands from the ischaemic penumbra are shown in the bar graphs; S — sham; M — middle cerebral artery occlusion; L — lithium chloride; D — Dickkopf-1. The results are expressed as the mean ± standard error of 5 animals in each group and were analysed by one-way ANOVA followed by the Student–Newman–Keuls test; *$p < 0.05$ vs. S group; #$p < 0.05$ vs. M group; †$p < 0.05$ vs. M+L group; ◊$p < 0.05$ vs. M+D group.

that the expression of Wnt-3a-positive cells was increased in the rats in the LiCl-administered group compared with rats in the nondrug administration group, whereas the inhibitor DKK1 hindered this neuroprotective effect ($F_{group} = 53.85$, $p < 0.05$; Fig. 6A, B). Furthermore, the mRNA levels of Wnt-3a, β-catenin, and Dvl-1 in the M group were higher than those in the S group. By contrast, the mRNA levels of GSK-3β and Axin-2 were lower in the LiCl group than those in the M group ($F_{group} = 63.87$, $p < 0.05$; Fig. 6D). LiCl treatment significantly augmented GSK-3β and Axin-2 mRNA expression and weakened Wnt-3a, β-catenin, and Dvl-1 mRNA expression compared to the M group ($F_{group} = 71.52$, $p < 0.05$; Fig. 6D). In the presence of DKK1 treatment, the mRNA expression levels of GSK-3β and Axin-2 were increased and the mRNA expression levels of Wnt-3a, β-catenin, and Dvl-1 were decreased compared to the M+L group ($F_{group} = 73.82$, $p < 0.05$; Fig. 6D). Thus, the Wnt signalling pathway blocker (DKK1) can inhibit the gene expression of Wnt-3a, Dvl-1, and β-catenin. Furthermore, the protein expression of GSK-3β and β-catenin, as

determined by western blot, confirmed that LiCl plays a neuroprotective role by activating the Wnt signalling pathway ($F_{group} = 64.74$, $p < 0.05$; Fig. 6C, E).

DISCUSSION

In this study, we apply the rat model with MCAO to simulate human ischaemia stroke. Administration of LiCl improved both impaired neurobehavioral ability and antioxidant capacity; it reduced the infarct volume, brain oedema, and inflammatory factors, which was corroborated by attenuated histopathologic changes. LiCl also increased the mRNA levels of Wnt-3a, β-catenin, and Dvl-1, and decreased the expression levels of mRNA of both GSK-3β and Axin-2 in ischaemic penumbra. However, these neuroprotective effects were reversed when pretreated with the Wnt signalling inhibitor DKK1.

Hippocampus is one of the most important brain areas relevant to learning and memory [15], some studies had found that the hippocampal CA1 area is particularly impressionable to ischaemic insult [15, 24]. Therefore, we choose neurons in the hippocampal CA1 area as the object of study. The MWM test is commonly used in the evaluation of cognitive function in rodents [24]. The training trial is applied to assess spatial or place learning, and the probe trials assess whether the animals remember the platform position. Current, experiment found that the S group rats quickly learned the platform location and rapidly arrived at the escape platform. On the contrary, the escape latency in the M group showed a significant increase, together with that of the distance travelled. The escape latency times after LiCl treatment showed an obvious decrease in both the M+L group and the M+L+D group, especially in the M+L group. Furthermore, a significant increase was noticed in the time going through the platform after LiCl treatment compared to the M group. However, a Wnt antagonist DKK1 suppressed the neuroprotective effects of LiCl treatment. These data demonstrated that LiCl improves impaired spatial learning and memory ability in MCAO rats.

The pathophysiology of stroke is related to many complex factors, including oxidative stress, injury, inflammatory response, and apoptosis. Oxidative stress is one of the mechanisms of cerebral ischaemia-reperfusion injury, and the production of ROS plays a major role in the early stage of cerebral ischaemia-reperfusion injury [16]. In the brain, intracellular antioxidants such as SOD, GSH-Px, and catalase can inhibit the production of oxygen free radicals and protect brain tissue from the cytotoxicity of ROS at an early stage. In addition, excess ROS can lead to lipid peroxidation, producing MDA, 4-hydroxynonenal and acrolein [2]. MDA is also an important marker of oxidative stress, causing more serious oxidative stress damage by destroying lipids, enzymes, and nucleic acids in cell membranes or organelles. Current, data indicate that LiCl treatment resulted in a significant decrease in the levels of MDA and ROS, and a remarkable increase in the levels of SOD and GSH-Px; however, the Wnt inhibitor DKK1 attenuates LiCl antioxidant activity. At the same time, the inflammatory response, involving inflammatory cells and inflammatory mediators, is one of the causes of exacerbation of ischaemic brain injury [14]. The major cytokines associated with inflammation in ischaemic brain injury include IL-1, TNF-α, and IL-6, which have been observed to be upregulated in ischaemic brain injury [36]. Among various inflammatory factors, TNF-α is the main proinflammatory factor and participates in the pathophysiological process of ischaemic brain injury [10, 27]. IL-6, which is produced by monocytes, macrophages, and endothelial cells, and it appears rapidly in the early stages of ischaemic brain injury and increases rapidly [10]. The present study confirmed that IL-6 and TNF-α were induced by ischaemia; however, which was downregulated following LiCl treatment. These findings are consistent with previous studies [33, 34]. It has been reported that LiCl has a protective effect on apoptosis of damaged neurons in ischaemic brain injury [34]. In this process, activation of apoptotic genes and inactivation of antiapoptotic genes were observed. Ischaemia can downregulate Wnt signalling pathway and inhibit the transcription of many target genes including apoptotic genes Bcl-2 and Bax [26, 34]. Current studies have shown that LiCl upregulates the antiapoptotic Bcl-2 molecule and downregulates the proapoptotic Bcl-2 associated X protein (Bax). The molecular and cellular effects of lithium may be to inhibit n-methyl-D-aspartate receptor, up-regulate cellular protective Bcl-2, and down-regulate pro-apoptotic Bax [19].

The Wnt signalling pathway is well known for nervous system development and synapse formation [38], it is associated with neuronal differentiation and development and migration [1, 37]. To there exist of Wnt signals, β-catenin is able to translocate to the nucleus [7]. Signalling activating through the binding of a Wnt to a Frizzled family receptor, low-density

lipoprotein receptors interrelated with the protein 5/6 coreceptor to form a ternary cell surface complex. Thereafter, the β-catenin complex of destructed, which was constituted of GSK-3β and 2 folding proteins, and the axis inhibition protein, is then disintegrated by relevant to Disheveld protein [32]. Wnt signalling pathway activation also contributed to functional recovery and increased neuroprotective processes and neurogenesis after cerebral ischaemia [22]. Abnormal alterations to key molecules in the Wnt signalling pathway, including Wnt-3a, Axin-2, and β-catenin, induce Wnt signalling pathway activation. Wnt signalling pathway activation also contributed to functional recovery and increased neuroprotective processes and neurogenesis after cerebral ischaemia [32]. As an antagonist of the Wnt pathway, DKK1 prevents Wnt activation by binding to the Wnt receptor, leading to the activation of the Wnt/β-catenin signalling pathway [23]. Recent studies showed that DKK1 is potentially induced in the hippocampal CA1 area after global cerebral ischaemia, and administration of DKK1 antisense oligonucleotides protects the CA1 region against cerebral ischaemia-induced neuronal death [40]. In this study, to further examine the direct effects of the drug on the outcomes of ischaemic stroke, rats received an intracerebroventricular injection of the inhibitor DKK1 before LiCl treatment. We found that LiCl significantly decreased Wnt-3a-positive cells, increased β-catenin positive cells, and promoted neural functional recovery. It upregulated the mRNA expression levels of Wnt-3a, β-catenin, and Dvl-1, and downregulated the mRNA expression levels of GSK-3β and Axin-2. Nevertheless, those changes were reversed in the presence of DKK1. Our findings are consistent with previous studies [20, 39].These results indicated that the neuroprotection of LiCl may be related to the activation of Wnt signalling pathway.

Our research provides new clues to explain the role of Wnt signalling pathway regulation in causing ischaemic stroke. Nevertheless, there were several limitations for this study. First, although we finding that LiCl treatment provided strong protection against focal cerebral ischaemia, we did not insight which other cell signalling pathways were involved in its neuroprotective mechanism. Second, one dose of lithium was used in our study, and the therapeutic time windows were not explored. Third, only the chronic effects of LiCl for 1 week after stroke were studied, which need to be further explored.

CONCLUSIONS

In conclusion, our current studies show that LiCl has neuroprotective effects on focal cerebral ischaemia injury, and this study mimics the clinical interventions available in most ischaemic stroke patients. The mechanism by which LiCl promotes neural restoration involves on the Wnt signalling pathway activation. Therefore, we believe that LiCl may be an indispensable therapeutic agent for nerve repair after ischaemic brain injury.

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