Involvement of the Wnt signaling pathway and cell apoptosis in the rat hippocampus following cerebral ischemia/reperfusion injury**

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Abstract
We investigated the role of the Wnt signaling pathway in cerebral ischemia/reperfusion injury by examining β-catenin and glycogen synthase kinase-3β protein expression in the rat hippocampal CA1 region following acute cerebral ischemia/reperfusion. Our results demonstrate that cell apoptosis increases in the CA1 region following ischemia/reperfusion. In addition, β-catenin and glycogen synthase kinase-3β protein expression gradually increases, peaking at 48 hours following reperfusion. Dickkopf-1 administration, after cerebral ischemia/reperfusion injury, results in decreased cell apoptosis, and β-catenin and glycogen synthase kinase-3β expression, in the CA1 region. This suggests that β-catenin and glycogen synthase kinase-3β, both components of the Wnt signaling pathway, participate in cell apoptosis following cerebral ischemia/reperfusion injury.

Key Words
neural regeneration; brain injury; Dickkopf-1; Wnt signaling pathway; cell apoptosis; β-catenin; glycogen synthase kinase-3β protein; cerebral ischemia/reperfusion injury; grant-supported paper; neuroregeneration

Research Highlights
(1) β-catenin and glycogen synthase kinase-3β protein expression are increased in the rat hippocampal CA1 region following ischemia/reperfusion.
(2) Dickkopf-1, a negative modulator of the Wnt pathway, suppresses β-catenin expression in the rat hippocampal CA1 region following cerebral ischemia/reperfusion.
(3) Dickkopf-1 suppresses glycogen synthase kinase-3β protein expression in the rat hippocampal CA1 region following cerebral ischemia/reperfusion.
(4) Dickkopf-1 reduces neuronal cell apoptosis in the rat hippocampal CA1 region following cerebral ischemia/reperfusion.

Abbreviations
DKK1, Dickkopf-1; GSK-3β, glycogen synthase kinase-3β

INTRODUCTION
Previous studies have predominantly focused on prevention of ischemic cerebrovascular disease damage to the central nervous system, by inhibiting cell apoptosis[1-2]. The Wnt signaling pathway plays an important role in formation of the central nervous system, proliferation and differentiation of neural stem cells[3], regulation of cell development and death[4], and participates in cerebral ischemia/hypoxia injury[5]. However, the effects...
of β-catenin and glycogen synthase kinase-3β (GSK-3β) on cell apoptosis following acute cerebral ischemia/reperfusion, have not been fully elucidated.

Dickkopf-1 (DKK1) is a negative modulator of the Wnt pathway, and therefore suppresses Wnt signaling⁶. DKK1 inhibits the Wnt pathway via upstream molecules, namely the frizzled and dishevelled proteins⁷. Our study examined the effect of DKK1 on cell apoptosis, and β-catenin and GSK-3β protein expression, in the hippocampal CA1 region of rats following acute cerebral ischemia/reperfusion. Our results confirm a relationship between the Wnt pathway and cerebral ischemia/reperfusion injury.

RESULTS

Quantitative analysis of experimental animals
A total of 66 rats were randomly assigned to three groups: sham surgery group (no middle cerebral artery occlusion, n = 6), model group (middle cerebral artery occlusion, n = 30) and DKK1 group (middle cerebral artery occlusion + DKK1 administration, n = 30). Six rats from each of the model and DKK1 groups were selected at 3, 6, 24, 48 and 72 hours following reperfusion for experimental analysis. Sham rats were obtained at 72 hours following reperfusion. A total of 66 rats were included in the final analysis.

DKK1 reduced cell apoptosis in the rat hippocampal CA1 region following cerebral ischemia/reperfusion
TUNEL staining in the sham surgery group was used to detect minimal apoptotic cells in the hippocampal CA1 region (not examined statistically). However, in the model and DKK1 groups, the number of apoptotic cells in the CA1 region increased at 3 hours and peaked at 48 hours, following ischemia/reperfusion. Compared with the model group, the number of apoptotic cells in the DKK1 group significantly decreased at various time points (P < 0.05 or P < 0.01; Table 2). The number of β-catenin-positive cells was also decreased in the DKK1 group, compared with the model group, but there was no significant difference (P > 0.05; Table 3).

Table 1 Dickkopf-1 effects on cell apoptosis (number in × 200 visual field) in the rat hippocampal CA1 region following cerebral ischemia/reperfusion injury

<table>
<thead>
<tr>
<th>Group</th>
<th>Reperfusion time (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Model</td>
<td>17.09±1.41</td>
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<tr>
<td>DKK1-1</td>
<td>15.33±1.63a</td>
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</table>

Results are expressed as mean ± SD of three rats in each group at each time point. aP < 0.05, bP < 0.01, vs. model group (t-test).

DKK1 inhibited β-catenin and GSK-3β protein expression in the rat hippocampal CA1 region following cerebral ischemia/reperfusion
Immunohistochemistry showed minimal β-catenin and GSK-3β expression in the sham surgery group (not examined statistically). In the model and DKK1 groups, β-catenin and GSK-3β expression was visible at various time points, and peaked at 48 hours following reperfusion (Figure 2). Compared with the model group, the number of GSK-3β-positive cells was significantly reduced, at corresponding time points, in the DKK1 group (P < 0.05 or P < 0.01; Table 2). The number of β-catenin-positive cells was also decreased in the DKK1 group, compared with the model group, but there was no significant difference (P > 0.05; Table 3).

Figure 1 Cell apoptosis in the rat hippocampal CA1 region (TUNEL staining, × 200).
Apoptotic cells with brown or yellow nuclei are visible in the model and Dickkopf-1 groups at various time points (arrows). Fewer apoptotic cells with weak staining are observed in the Dickkopf-1 group, compared with the model group, at corresponding time points.
Western blot analysis confirmed our immunohistochemistry results, with decreased β-catenin (P > 0.05; Figure 3, Table 4) and significantly decreased GSK-3β (P < 0.05 or P < 0.01; Figure 3, Table 5) protein expression, at each time point, in the DKK1 group, compared with the model group.

Table 2  Dickkopf-1 effects on glycogen synthase kinase-3β (GSK-3β) protein expression (number in × 200 visual field) in the rat hippocampal CA1 region following cerebral ischemia/reperfusion injury

<table>
<thead>
<tr>
<th>Group</th>
<th>Reperfusion time (hour)</th>
<th>3</th>
<th>6</th>
<th>24</th>
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<tr>
<td>Model</td>
<td>9.43±1.89</td>
<td>9.96±1.07</td>
<td>10.89±1.57</td>
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<tr>
<td>Dickkopf-1</td>
<td>8.40±1.23</td>
<td>8.60±1.02</td>
<td>8.86±1.63</td>
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<table>
<thead>
<tr>
<th>Group</th>
<th>Reperfusion time (hour)</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>12.12±0.88</td>
<td>10.24±1.65</td>
<td></td>
</tr>
<tr>
<td>Dickkopf-1</td>
<td>9.17±1.29</td>
<td>7.78±1.39</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD of three rats in each group at each time point. *P < 0.05, **P < 0.01, vs. model group (t-test).

DISCUSSION

The process of cell apoptosis following cerebral ischemia is complex, and is regulated by many proteins and genes[1]. β-catenin is at the center of the Wnt signaling pathway, and its activation or degradation leads to changes in Wnt signaling[8-9]. GSK-3β has a regulatory effect on cell growth, differentiation, mutation and apoptosis, and is involved in many signaling pathways, participating predominantly in negative regulation of signals[10]. Previous studies have shown that upregulation of GSK-3β activity can activate Bax, c-jun and the caspases family, in normal histiocytes and various tumor cells[11-12], resulting in cell apoptosis. Increased GSK-3β activity promotes cell apoptosis following myocardial ischemia and cerebral ischemia/reperfusion[13-14].
Our results confirm that cell apoptosis increases, along with increased β-catenin and GSK-3β protein expression, peaking at 48 hours in the rat hippocampal CA1 region following cerebral ischemia/reperfusion. This suggests that cerebral ischemia/reperfusion injury activates β-catenin and GSK-3β. Moreover, increased expression of β-catenin and GSK-3β may induce cell apoptosis.

DKK1, an important regulatory factor in the Wnt signaling pathway, prevents the binding of Wnt to the low-density lipoprotein receptor related protein 5/6 and the Frz receptor, causing decreased intracellular β-catenin levels, and thereby inhibiting activation of the Wnt signaling pathway. Shou et al. found DKK1 expression was negatively associated with cell apoptosis. Our results demonstrate that following DKK1 administration, cell apoptosis was reduced, and β-catenin and GSK-3β protein expression decreased, in the rat hippocampal CA1 region following cerebral ischemia/reperfusion injury. Overall, we show that β-catenin and GSK-3β participate in cell apoptosis following cerebral ischemia/reperfusion injury, indicating that modifying the Wnt signaling pathway offers a new route to manage cerebral ischemia/reperfusion injury.

**MATERIALS AND METHODS**

**Design**

This is a randomized controlled animal study.

**Time and setting**

Experiments were performed at the Experimental Center, Hebei United University, China from March 2010 to October 2011.

**Materials**

A total of 66 clean male Sprague-Dawley rats, aged 2–4 months and weighing 200–250 g, were supplied by the Laboratory Animal Institute, Chinese Academy of Medical Sciences (license No. SCXK (Jing) 2010-0013). The rats were housed in the Animal Laboratory of Environmental Barriers, Hebei United University, China. They were kept in a temperature controlled environment (23 ± 2°C) with natural illumination, and were acclimatized for 2 weeks before experiments. The protocols were conducted in strict accordance with the **Guidance Suggestions for the Care and Use of Laboratory Animals**, formulated by the Ministry of Science and Technology of China.
Methods

Establishment of cerebral ischemia/reperfusion models

In accordance with a modified Longa's method\textsuperscript{[18]}, the rat model of middle cerebral artery occlusion was established. The rats were fasted for 12 hours with no water for 4 hours prior to surgery. The rats were intraperitoneally anesthetized with 10% chloral hydrate (0.3 mL/kg), and laid in the supine position. A longitudinal incision (approximately 25 mm) was made on the neck to expose the right common carotid artery. The proximal part of the common carotid artery and bifurcation of the external carotid artery were ligated, and the external carotid artery was reversely straightened. A cut was made about 5 mm from the end of the common carotid artery. A 0.26-mm diameter fishing line was inserted along the internal carotid artery, and withdrawn 0.5 mm after meeting resistance. The depth of penetration was approximately 18.5 ± 0.5 mm. The artery was ligated at the proximal part. The incision was sutured using a full layer suture. After 2 hours, the fishing line was pulled out to enable reperfusion. The surgery was performed at 22 ± 2°C. Inclusion criteria\textsuperscript{[18]} were as follows: rats turned left after consciousness; and no spasticity or defecation occurred during the first 3 hours after surgery. The rats were separated into four groups: sham, surgery, DKK1, and DKK1+Dkk1. The DKK1 group was administered recombinant human DKK1 (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), at a concentration of 1.0 mg/mL, was injected into the lateral ventricle (Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd., Beijing, China), with sections developed using 3,3′-diaminobenzidine (Maixin-Bio, Fuzhou, Fujian Province, China), and counterstained in hematoxylin. Cell apoptosis in the rat hippocampal CA1 region was observed under the light microscope (Olympus, Tokyo, Japan). Positive cells were quantified using a CMIAS real-color automatic image analyzer system (Air Force General Hospital-Biomedical Engineering Institute, Beihang University, Beijing, China). Six fields from each section were observed under × 200 magnification, and the number of positive cells in each field counted, obtaining an average value. Nuclei with brown particles represented TUNEL-positive cells.

β-catenin and glycogen synthase kinase-3β immunohistochemistry in the rat hippocampal CA1 region

Immunohistochemistry was performed in accordance with a kit (Maixin-Bio). Sections were blocked in normal goat serum, incubated with rabbit anti-β-catenin or GSK-3β polyclonal antibodies (both 1:175; Beijing Biosynthesis Biotechnology Co., Ltd.) at 4°C overnight, followed by incubation in biotinylated goat anti-rabbit IgG (1:200; Maixin-Bio) and horseradish peroxidase-labeled streptavidin (Maixin-Bio). Antibody signals were detected by 3,3′-diaminobenzidine coloration. Sections were examined using a light microscope. Positive cells were quantified using the CMIAS real-color automatic image analyzer system. Cells with brown cytoplasm represented positive cells. Six non-overlapping fields of the hippocampal CA1 region from each rat were quantified under × 200 magnification, with the average value calculated.

Western blot analysis of β-catenin and glycogen synthase kinase-3β protein expression in the rat hippocampal CA1 region

Hippocampal tissue homogenates were collected for protein quantification. Protein was isolated by SDS-PAGE. The specimens were incubated with rabbit anti-GSK-3β (1:175) and β-catenin (1:175) polyclonal antibodies at 4°C overnight, then incubated in biotinylated goat anti-rabbit IgG (1:300) at room temperature for 0.5 hours, followed by streptavidin-horseradish peroxidase complex (Maixin-Bio) at room temperature for 0.5 hours, and then 3,3′-diaminobenzidine coloration. Absorbance values were measured using the CMIAS real-color automatic image analyzer system, under the same conditions for each antibody. The experiment was repeated in triplicate, with β-actin used as an internal reference.

Preparation of tissue specimens

Six rats from each group were collected at corresponding time points. Three rats were sacrificed and perfusion fixed in paraformaldehyde. The right cerebral hemisphere was dissected. Tissue sections (2 mm) were obtained from the region 2 mm posterior to the optic chiasma, and fixed, dehydrated in alcohol, permeabilized with xylene, immersed in wax, embedded and serially sliced into 5 μm coronal sections. These sections were used for TUNEL staining and immunohistochemistry. The remaining three rats from each group, were deeply anesthetized with 10% chloral hydrate (0.3 mL/100 g) and then decapitated. The brain was removed on ice and stored at 4°C for western blot analysis.

TUNEL staining to determine cell apoptosis in the rat hippocampal CA1 region

TUNEL staining was performed in accordance with a kit (Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd., Beijing, China), with sections developed using 3,3′-diaminobenzidine (Maixin-Bio, Fuzhou, Fujian Province, China), and counterstained in hematoxylin. Cell apoptosis in the rat hippocampal CA1 region was observed under the light microscope (Olympus, Tokyo, Japan). Positive cells were quantified using a CMIAS real-color automatic image analyzer system (Air Force General Hospital-Biomedical Engineering Institute, Beihang University, Beijing, China). Six fields from each section were observed under × 200 magnification, and the number of positive cells in each field counted, obtaining an average value. Nuclei with brown particles represented TUNEL-positive cells.

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**Statistical analysis**

Data were managed using Excel and analyzed using SPSS 17.0 (SPSS, Chicago, IL, USA). All values were expressed as mean ± SD. The mean values at various time points from the model and DKK1 groups were compared using t-tests. A value of P < 0.05 was considered statistically significant.

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**Author contributions:** Bin Liu and Jing Tang participated in study concept and design, and wrote the manuscript. All authors participated in study implementation and data analysis. Jing Tang was in charge of data management and statistical analysis. Bin Liu was responsible for manuscript authorization and obtained funding. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Ethical approval:** This study was approved by the Animal Ethics Committee, Affiliated Hospital of Hebei United University, China.

**Author statements:** The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

**REFERENCES**


(Talked by Yan JH, Zhao R/Qiu Y/Song LP)