Cell apoptosis and proliferation in rat brains after intracerebral hemorrhage: role of Wnt/β-catenin signaling pathway

Ling ZHOU¹**, Li DENG²**, Neng Bin CHANG³, Ling DOU³, Chao Xian YANG²*

¹Department of Endocrinology, First Affiliated Hospital, Luzhou Medical College, Luzhou, P.R. China
²Department of Neurobiology, Research Center for Preclinical Medicine, Luzhou Medical College, Luzhou, P.R. China
³Department of Anatomy, School of Basic Medical Sciences, Luzhou Medical College, Luzhou, P.R. China

Background/aim: To investigate the role of Wnt/β-catenin signaling pathway in cell apoptosis and proliferation in intracerebral hemorrhage (ICH) in rats.

Materials and methods: The ICH rats were established by stereotaxic infusion of 50 µL autologous arterial blood into the right striatum. Pathological characteristics of brain tissue was assessed by hematoxylin and eosin and TUNEL staining, and the expressions of proliferating cell nuclear antigen (PCNA) and Wnt3a/β-catenin/cyclin D1 were investigated by immunohistochemistry or reverse-transcription polymerase chain reaction.

Results: The number of apoptotic cells and PCNA-positive cells kept increasing from the day following the ICH induction and reached a peak on the 3rd and 14th days, respectively. Some of the PCNA-positive cells could coexpress neurofilament protein-200 or glial fibrillary acidic protein. Wnt3a, β-catenin, cyclin D1, and PCNA mRNAs reached maximal expression levels on the 3rd, 7th, 7th, and 14th days, respectively. Moreover, the number of apoptotic cells was significantly positively correlated with the expressions of Wnt3a and β-catenin mRNAs, and negatively correlated with the number of PCNA-positive cells.

Conclusion: Our results suggest that the Wnt/β-catenin signaling pathway is associated with cell apoptosis and expression of PCNA in the ICH rat brain and regulates the balance between cell apoptosis and proliferation.

Key words: Intracerebral hemorrhage, rat brain, cell apoptosis, proliferating cell nuclear antigen, Wnt/β-catenin signaling pathway

1. Introduction
Previous studies have indicated that intracerebral hemorrhage (ICH) induces neurological damage due to local tissue deformation and the subsequent developments of excitotoxicity, inflammation, and apoptosis (1,2). ICH also induced neurogenesis, which is helpful to promote functional recovery (3–5).

The Wnt/β-catenin signaling pathway is one of several key conserved intercellular signaling pathways in vertebrate animals, which plays important roles in the proliferation, differentiation, and function of many cell and tissue types (6,7). However, the relation between cell apoptosis, expression of proliferating cell nuclear antigen (PCNA, a marker of cell proliferation), and Wnt/β-catenin signaling pathway in brains subjected to ICH is not well understood. Therefore, the present study aimed to investigate the number of apoptotic cells and the expression levels of PCNA and Wnt3a/β-catenin/cyclin D1 in the peripheral hemorrhagic region to prove the role of the Wnt/β-catenin signaling pathway in cell apoptosis and proliferation in the ICH brain.

2. Materials and methods
2.1. Animals and treatment
The study involved 56 male Sprague-Dawley rats (Laboratory Animal Center of Luzhou Medical College, Luzhou, China), with a body mass of 200–250 g each. The rats were housed under conditions of constant temperature, humidity, and a 12/12-h light/dark cycle, with food and water available. The animals were randomly divided into 3 groups: normal group (n = 8), sham-operation group (n = 8), and ICH group (n = 40). The experimental protocol was in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by

* Correspondence: lyycx@foxmail.com
** These authors contributed equally to the article.
the Ministry of Science and Technology of the People’s Republic of China.

2.2. Operative procedure
ICH was induced by autologous blood injection as described previously (8). Briefly, rats were anesthetized with pentobarbital (30 mg/kg) and positioned in a stereotaxic frame (Xi’an Northwest Optical Instrument Factory, China). Burr holes were drilled, 50 µL of autologous arterial blood was injected into the right striatum (coordinates: 0.2 mm anterior, 6 mm ventral, and 3 mm lateral to bregma) at 10 µL/min. The rats in the ICH group were subdivided into 5 subgroups based on different time points (1–30 days) after operation (8 rats for each time point). The rats in the sham-operated group were subjected to the same procedure, but no blood was injected. The rats in the normal group were not treated.

Three rats from each subgroup of the ICH group and from the normal and sham-operated groups were euthanized and decapitated for total RNA extraction. The remaining 5 rats were treated with successive cardiac perfusion of physiological saline and 4% paraformaldehyde, followed by paraffin embedding. Subsequently, 5-µm-thick coronal sections were taken and used for follow-up experiments.

2.3. H&E and TUNEL staining
Hematoxylin and eosin (H&E) staining was used to observe histological features (9). TUNEL staining was employed to identify the number of cells exhibiting DNA fragmentation under light microscope. Staining of apoptotic cells was conducted according to the manufacturer’s instructions (Roche, Switzerland) as previously described (10). Briefly, paraffin sections were dewaxed and hydrated by a gradient of alcohol to water, and were then treated with 3% H2O2 and proteinase K. After digestion with proteinase K, each sample was incubated with 50 µL of TUNEL reaction mixture and then with converter-peroxidase, and developed with 3,3’-diaminobenzidine (DAB). Negative controls were performed using label solution instead of TUNEL reaction mixture in the process. The sections were counterstained with hematoxylin.

2.4. RT-PCR
Total RNA was isolated from homogenates (80 mg) of the right striatum using TRIzol Reagent (Invitrogen, USA). All gene segments were amplified and verified by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR), conducted using the Takara One-Step RNA PCR kit (Takara, China). Negative controls were performed by omitting the template. The products of PCR were checked by 2% agarose gel electrophoresis, and the expression abundance of each mRNA was detected and compared to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The sequences of primers were designed and are shown in Table 1.

2.5. Immunohistochemistry
Expression of PCNA protein in brain tissue was detected with immunohistochemical staining. Briefly, sections were dewaxed, rehydrated, and digested with 0.1% trypsin, then blocked in normal 10% goat serum. After being incubated with monoclonal primary antibody against PCNA (1:100 dilution; Wuhan Boster Co., China) at 4 °C overnight, the sections were treated with biotinylated goat antimouse IgG (1:100 dilution; Wuhan Boster Co.) for 30 min at 37 °C. After sequential incubation with HRP-streptavidin (1:100 dilution; Wuhan Boster Co.) at 37 °C for 30 min, the sections were treated with DAB to display PCNA-positive cells. Negative control sections were identically stained, except that the primary antibody was omitted. Hematoxylin was also used in counterstaining of the sections.

In order to investigate the fate of PCNA-positive cells, double immunohistochemical staining was carried out using a method based on the instruction manual of the double staining kit (Zhongshan Goldenbridge, China). Briefly, PCNA was labeled by the previously described procedure of immunohistochemistry, but DAB was replaced with 3-amino-9-ethyl carbazole. After PCNA staining, the sections were sequentially incubated with a double staining enhancer, monoclonal primary antibody against neurofilament protein-200 (NF-200, 1:100 dilution; Wuhan Boster Co.), or polyclonal primary antibody against glial fibrillary acidic protein (GFAP, 1:100 dilution; Sigma, USA), biotinylated IgG, and AP-streptavidin. 5-Bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium was then used as chromogen for light microscopy.

2.6. Image analysis and statistical analysis
The number of apoptotic cells and immunoreactive cells in the coronal section from the bregma (0.3 to 1.2 mm) was counted in 3 to 4 sections per animal (n = 5), and positive

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA-F</td>
<td>GCT CTG AGG TAC CTG AAC T</td>
</tr>
<tr>
<td>PCNA-R</td>
<td>GCC TAA GAT CCT TCT TCA TC</td>
</tr>
<tr>
<td>Wnt3a-F</td>
<td>GAG TCT CGT GGC TGG GTG GA</td>
</tr>
<tr>
<td>Wnt3a-R</td>
<td>TTG GGC TCG CAG AAG TTA GG</td>
</tr>
<tr>
<td>β-catenin-F</td>
<td>GAC ACC TCC CAA GTC CTT TAT G</td>
</tr>
<tr>
<td>β-catenin-R</td>
<td>GTA CAA CGG GCT GTT TCT ACG</td>
</tr>
<tr>
<td>Cyclin D1-F</td>
<td>GCG TAC CCT GAC ACC AAT C</td>
</tr>
<tr>
<td>Cyclin D1-R</td>
<td>TCC TCG CAG ACC TCT AGC AT</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>CCT CAA GAT TGT CAG CAA T</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>CCA TCC ACA GTC TTC TGA GT</td>
</tr>
</tbody>
</table>
cells were measured on 5 nonoverlapping regions within the right striatum per section under a 40× objective lens (Olympus, Japan). All analyses were performed using SPSS 12.0 for Windows. Data are presented as the mean ± standard error (SE), and statistical comparisons were made by t-tests and ANOVA. Differences were considered significant when P < 0.05.

3. Results

3.1. Histological characteristics of brain tissue
H&E staining shows the brain tissues of the normal and sham-operated groups (Figures 1A and 1B). In the ICH group, oval or irregularly shaped hematomas were observed in the ipsilateral striatum on the first day. In addition, obvious edema appeared in the perihematomal region and the neural tissues seemed loosely packed, allowing infiltration of neutrophils and settlement of migrating blood cells (Figure 1C). At 3 days after ICH, hematomas were still clearly visible in the lesion area, and lysis of certain blood cells and hemosiderin deposition were also observed. Moreover, edema persisted in the area surrounding the hematomas, accompanied by neuron swelling and nuclear fragmentation, dissolution or disappearance; some gitter cells were also observed (Figure 1D). At 7 days after ICH, blood cell lysis further increased in the hemorrhagic focus. Partial cerebral tissue surrounding the hematomas underwent colliquation and necrosis, and capillary vessels and hyperplasia of astrocytes were clearly visible (Figure 1E). At 14–30 days after ICH, most blood cells had disintegrated and subsequently disappeared, leading to the formation of oval or irregularly shaped capsular space (Figure 1F).

3.2. Cell apoptosis
In the normal and sham-operated groups, 0–4 TUNEL-positive cells were present in the striatum (Figure 2A). From 1 to 30 days after ICH, the TUNEL-positive cells were mainly present in the striatum, frontoparietal cortex, and hippocampus. In the ICH group, the number of TUNEL-positive cells was increased on the 1st day (58.7 ± 4.8), peaked on the 3rd day (98.2 ± 7.9), and then declined onward, but on the 30th day was significantly more than that of the normal or sham-operated group (Figures 2B, 2C, and 3).

3.3. Distribution and fate of PCNA-positive cells
PCNA-positive cells were mainly distributed in the subependymal ventricular zones and choroid plexus in the normal and sham-operated groups (Figure 4A). From 1 to 30 days after ICH, the PCNA-positive cells were mainly present in the subependymal ventricular zones, striatum, frontoparietal cortex, corpus callosum, and choroid plexus (Figures 4B–4F). In the ICH group, a large number of PCNA-positive cells were identified in the periphery of the hemorrhagic focus in striatum on the 1st day (37.2 ± 4.4), and the number of PCNA-positive cells reached their peak on the 14th day (58.5 ± 5.3), followed by a gradual decline (Figure 5).

Figure 1. Histological features of rat brains (H&E staining). A) The brain tissue of the normal group. B) A needle track in the striatum in the sham-operated group. C) A visible hematoma in the striatum of the ICH group on the 1st day. D) Gitter cells (black arrow) are shown in the ICH group on the 3rd day. E) Colliquation tissue (triangle), capillary vessels (arrow), and astrocytes (pentagon) are shown in the ICH group on the 7th day. F) A capsular space is shown in the striatum in the ICH group on the 14th day. Bar = 50 µm.
Figure 2. Apoptotic cells in rat brains (TUNEL staining). A) Apoptotic cells in the striatum of the sham-operated group. B) Apoptotic cells in the striatum of the ICH group on the 3rd day. C) Apoptotic cells in the striatum of the ICH group on the 30th day. Bar = 50 µm.

Figure 3. The change in the number of apoptotic cells in rat brains. N and sham refer to the normal group and sham-operated group, respectively, and 1d–30d represent different time points of the ICH group. *: Compared with the normal and sham-operated groups (P < 0.01), #: compared with the preceding time point of the ICH group (P < 0.05).

Figure 4. Distribution of PCNA-positive cells by immunohistochemistry in rat brains. A) PCNA-positive cells were observed in the subventricular zone of the sham-operated group. B–F) In the ICH group, PCNA-positive cells were found in the subependymal ventricular zones (B), striatum (C), frontoparietal cortex (D), corpus callosum (E), and choroid plexus (F). Bar = 50 µm.
Double immunohistochemical staining indicated that PCNA/GFAP double-positive cells were widely distributed in the striatum, frontoparietal cortex, and subependymal ventricular zone (Figures 6A–6C); on the other hand, PCNA/NF-200 double-positive cells were mainly distributed in the frontoparietal cortex (Figure 6D).

3.4. Expressional levels of Wnt3a, β-catenin, cyclin D1, and PCNA mRNAs

The results of agarose gel electrophoresis of representative PCR reaction products are shown in Figure 7A. The expression of each gene was normalized to that of the housekeeping gene GAPDH. Figure 8 shows that Wnt3a,
β-catenin, cyclin D1, and PCNA mRNAs reached maximal expression levels on the 3rd, 7th, 7th, and 14th days, respectively.

3.5. Correlation analysis of various experimental parameters
Correlation coefficients of various experimental parameters are shown in Table 2. The expressions of Wnt3a, β-catenin, and cyclin D1 mRNAs are significantly positively correlated with the expression of PCNA mRNA, and the latter is obviously positively correlated with the number of PCNA-positive cells. The number of apoptotic cells is also significantly positively correlated with the expressions of Wnt3a and β-catenin mRNAs, but negative correlated with the number of PCNA-positive cells.

4. Discussion
In this study we observed that cerebral tissue in the periphery of the hemorrhagic focus underwent colliquation and necrosis. Hyperplasia of astrocytes and capillary vessels was also observed. Absorption of
blood cells may lead to the formation of small cavities in the hematoma site, which gradually merge to form a substantial capsular space. Our results relate to secondary brain injury that is triggered by neutrophil infiltration and microglia activation in the hematoma and its surrounding area (11).

The Wnt family of secreted glycoproteins can activate different intracellular signaling pathways and Wnt proteins comprise 19 family members that exist in mammalian genomes (12,13). Wnt3a, an important member of the Wnt family, can interact with cell membrane-associated proteins and induce the accumulation of β-catenin. Some experimental evidence shows that accumulation or overexpression of Wnt and/or β-catenin could induce apoptosis in melanoma and hepatocellular carcinoma cells (14–16). Cell apoptosis in the perihematoma region may be involved in the process of secondary injury (17,18). In the present study, both the expression levels of Wnt3a/β-catenin mRNA and the number of apoptotic cells rose in rat brains after ICH, and the expressions of Wnt3a and β-catenin mRNA were significantly positively correlated with the number of apoptotic cells. The data imply that activation of Wnt/β-catenin signaling may also induce apoptosis in rat brains after hemorrhagic stroke.

Activation of Wnt signaling leads to β-catenin nuclear translocation, followed by transcriptional activation of target genes in the nucleus, and cyclin D1 is a primary transcriptional target of β-catenin signaling (19–21). In this study, we noticed that expression levels of Wnt3a/β-catenin/cyclin D1 mRNAs were temporally and spatially associated with expression of PCNA mRNA, and the latter was obviously correlated with the number of PCNA-positive cells. The results imply that activation of the Wnt/β-catenin signaling pathway in the ICH brain could play an important role in regulating cell proliferation. In addition, considering the changes in cell apoptosis and expressions of PCNA and Wnt/β-catenin signaling and their relationship, we can suppose that the Wnt/β-catenin signaling pathway regulates the balance between cell apoptosis and proliferation.

Proliferation and differentiation of neural precursor cells (NPCs) might have a function in reconstructing the morphologic structures and functional activity during brain rehabilitation following stroke (22,23). Our previous study has shown that NPCs could migrate towards the regions distal to the hemorrhagic focus in ICH rats (8). In the present study we found that some of the PCNA-positive cells coexpressed NF-200 (neuron marker) and GFAP (astrocyte marker). Interestingly, we noted that PCNA/GFAP double-positive cells were widely distributed in the striatum, frontoparietal cortex, and subependymal ventricular zone, whereas PCNA/NF-200 double-positive cells were mainly distributed in the frontoparietal cortex. The results suggest that PCNA-positive cells may differentiate into glial cells and neurons, and the exact cell type is closely associated with the microenvironment.

In conclusion, Wnt/β-catenin signaling pathway may be associated with cell apoptosis and expression of PCNA in the ICH brain, furthermore regulating the balance between apoptosis and expression of PCNA. The exact differentiated cell type of PCNA-positive cells is closely associated with the microenvironment.

Acknowledgments
This work was supported by the Science Foundation of the Sichuan Province Educational Commission of China (#12ZA077) and the Application Basic Research Project of Sichuan Province of China (#2013JY0075).

References


