Effect of Qingxinkaiqiao compound on cortical mRNA expression of the apoptosis-related genes Bcl-2, BAX, caspase-3, and Aβ in an Alzheimer’s disease rat model

Hu Haiyan, Wang Yiyu, Zhang Yihui, Wang Wenhua, Xu Dongmei, Chen Zhiyu, Zhang Xiaoyan, Mao Dandan

OBJECTIVE: To investigate the effects of Qingxinkaiqiao (QK) compound in a rat model of Alzheimer’s disease induced with β-amyloid (Aβ) 1-40.

METHODS: Fifty-six three months, male, Sprague-Dawley rats were randomly divided into seven groups: blank control group, surgery group, model group, low-dose QK group, middle-dose QK group, high-dose QK group, and Aricept (donepezil hydrochloride) group, with eight rats in each group. Apart from the control and surgery groups, an Alzheimer’s disease model was established in all groups by bilateral hippocampal injection of Aβ 1-40. The surgery group received an injection of the same volume of physiological saline. Two days after model establishment, rats from the drug groups were administered the corresponding drugs; the control group and model group were administered an equal volume of physiological saline for 14 days. After treatment, real-time quantitative polymerase chain reaction, immunohistochemistry, and western blot assay were employed to confirm mRNA and protein expressions of Bcl-2, Bax, caspase-3, and Aβ, respectively.

RESULTS: Compared with the model group, Bcl-2 expression increased and Bax, caspase-3, and Aβ expression decreased in each drug treatment group (P < 0.05, P < 0.01). The expressions of middle-dose QK group were more significant than the high- and low-dose QK groups (P < 0.01, P > 0.05).

CONCLUSION: QK treatment resulted in significantly up-regulated Bcl-2 expression, down-regulated Bax, caspase-3, and Aβ expression, and reduced numbers of apoptotic cells in the cortex.
creased attention to this devastating disease. There remains no effective modern treatment for Alzheimer’s disease, but Traditional Chinese Medicine provides a unique therapeutic perspective for dementia. Qingxingkaigao (QK) compound from the "Fumanjian" is a Chinese medicine recipe documented in the medical book Jingyue Quanshu, written by Zhang Jing-yue during the Ming Dynasty. It has been used in clinical practice for many years. It can significantly improve cognitive dysfunction, as well as behavioral and psychological symptoms in patients. Previous results from our group showed that QK improves learning and memory in AD rats and decreases apoptosis in the hippocampal region. The present study aimed to investigate the effects of QK on cortical expression of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), cysteiny1 aspartate specific proteinase-3 (Caspase-3), and β-amyloid precursor protein (Aβ) in a rat model of AD induced by Aβ1-40.

MATERIALS AND METHODS

Experimental animals
Fifty-six male, specific pathogen-free, 3-month-old Sprague-Dawley (SD) rats weighing (250 ± 20) g were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. [Certification No. SCXK (Beijing) 2012-0001]. Rats were bred at the Wenzhou Medical University Laboratory Animal Center (clean experimental and standard animal feeding conditions). The rats were housed in a room with a 12-h light/dark cycle. The animals were subjected to experimentation after acclimatization for 1 week at 23-25 °C and relative humidity of 55% ± 5%, with free access to standard food and water. All experimental conditions followed ethical requirements related to experimental animals.

Drugs and reagents
The following herbs were purchased from the Dispensary of Traditional Chinese Medicine (Second Affiliated Hospital of Wenzhou Medical University): Dihuang (Radix Rehmanniae) 6 g, Baishao (Radix Paronae Alba) 6 g, Shichangpu (Rhizoma Acori Tatarinowii) 6 g, Maidong (Radix Ophiopogonis Japonici) 6 g, Mudanpi (Cortex Moutan Radicis) 6 g, Fushen (Porzia Cum Radix Pini) 6 g, Kushen (Radix Sophorae Flavescentii) 6 g, Shihu (Herba Dendrobii Nobilis) 6 g, Chenpi (Pericarpium Citri Reticulatae) 4 g, and Zhihu (Rhizoma Anemarrhenae) 5 g. The raw herbs were decocted with appropriate amounts of water, extracted two times, filtered and concentrated to drug stocks of 1 g/mL (crude drug), and stored at 4 °C. Donepezil (Eisai Pharmaceutical, Suzhou, China; batch number: 100223A) was made into a water suspension of the designed concentration prior to administration.

Reagents (sources) used in the study were as follows: Aβ1-40 and DMSO were purchased from Sigma (St. Louis, MO, USA); DAB chromogenic reagent kits were purchased from Zymed (San Diego, CA, USA); Hematin dye solution was purchased from FIR Biological (Beijing, China); Trizol Reagent was purchased from ShengGong Biological Engineering (Shanghai, China); Reverse transcriptase, fluorescence quantitative Ploymerase Chain Reaction (PCR) and SYBR green I were purchased from Bioneer (Daejeon, Korea). The quantitative PCR primers were purchased from Dalian Treasure Biological Engineering (Dalian, China); rabbit anti-rat BAX actin antibody, rabbit anti-rat Bcl-2 actin antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody were purchased from Cell Signal Technology (Beverly, MA, USA); rabbit anti-rat caspase-3 antibody and rabbit anti-rat Aβactin antibody were purchased from Bio wisdom Technology (St. Louis, MO, USA); chloral hydrate was purchased from Sinopharm Chemical Reagent (Shanghai, China); BCA protein assay kit and enhanced chemiluminescence kit were purchased from Pierce (Rockford, IL, USA).

Establishment of animal model and grouping
Aβ1-40 was incubated according to the manufacturer’s instructions to allow the change in an assembly state of the peptide with ensuing toxicity. Fifty-six rats were used in the experiment. Except for the randomly chosen 8 rats that served as the normal controls, the remaining 48 rats were sedated with 10% chloral hydrate (3-4 mL/kg) by intraperitoneal injection. The hair on the skull was shaved and the skin was disinfected with 75% alcohol. The skin on the skull was then incised and the periosteum was removed. Referring to the Paxinos and Watson Rat Brain Atlas, the bregma served as the 0 point. Then, 3 mm ventral from bregma, a cranial drill was employed to drill a hole through the skull 1.5 mm to the left and right of the midline. A needle was inserted 3 mm deep and a microsyringe was used to infuse 2 μL double-distilled H2O bilaterally into the hippocampus of the sham-surgery group. The remaining groups were injected with 2 μL Aβ1-40 at a concentration of 2.5 μg/μL (equivalent to 5 μg Aβ) and a speed of 0.5 μL/min. The needle was then held in place for 10 minutes after infusion to prevent leakage. Once the needle was removed, the scalp was sutured and iodine was used to disinfect the incision. Except for the normal control group (0 + NS) and the sham-surgery group (NS + NS), the remaining 40 successfully established model rats were randomly divided into five groups according to a random number sequence generated by a computer, with eight rats in each group: model control group (Aβ + NS), positive control group (Aβ + Aricept) treated with Aricept [1.67 mg·kg^-1·d^-1], and three QK-treated groups (Aβ + L-FJ/Aβ + M-FJ/Aβ + H-FJ) treated respectively with QK at various dosages according to the equivalent
adult human clinical dose (low-dose QK: 4.75 mg·kg⁻¹·d⁻¹; middle-dose QK: 9.5 mg·kg⁻¹·d⁻¹; high-dose QK: 19 mg·kg⁻¹·d⁻¹. Starting the following day, the rats were intragastrically administered the respective QK dose place for 14 days at a volume of 10 mg·kg⁻¹·d⁻¹ (starting at 10 o’clock in the morning every day). The control and model groups were given equivalent volumes of normal saline solution.

**Cortex sample handling**

At the end of the experiment, the rats were anaesthetized with 10% chloral hydrate (3-4 mL/kg) via intraperitoneal injection. The rats were then connected to the infusion apparatus, and the chest was opened along both sides of the sternum avoiding large blood vessels to expose the heart. A needle was inserted into the tip of the heart and into the aorta. The needle was then fixed with a vascular clamp and the right atrial appendage was incised. Cold saline (250 mL) was infused until the liver was clear, which was followed by 250 mL cold (4 °C) paraformaldehyde. The brains were quickly removed and further fixed in 4% paraformaldehyde. The cortex was rapidly separated from the brain and stored at −80 °C.

**Quantification of mRNA expression of Bcl-2, Bax, caspase-3, and Aβ in the cortex using real-time quantitative PCR**

Measurement of concentration and integrity detection of total RNA extraction. Total RNA was extracted according to Trizol kit instructions. RNA integrity was tested by separating the RNA by 5% agarose gel electrophoresis. The RNA solution was diluted and zeroed with diethylpyrocarbonate water (DEPC water). The OD values were obtained by ultraviolet spectrophotometer to determine RNA concentration. Synthesis of cDNA. A mixture of 1.0 μL (1.0 μg/μL) template total RNA, 2.0 μL (T18, 10 pmol/μL) and 2.0 μL (10 mmol/L) dNTP mix was placed into a centrifuge tube (0.5 mL) and water was added to a total of 15.0 μL. After mixing, the RNA was denatured for 10 min at 25 °C. The sample was centrifuged to collect the solution at the bottom of the centrifuge tube, and then 4.0 μL 5× reaction buffer, 1.0 μL RNase inhibitor, and 1.0 μL M-MLV RT were added to the sample, water was added to a total of 25.0 μL. The reaction was extended at 42 °C for 60 min, maintained at 85 °C for 5 min, and then terminated. The synthesized cDNA was stored at −20 °C.

Design of PCR primer. Primers for Bcl-2 mRNA, Bax mRNA, caspase-3 mRNA, and Aβ mRNA were designed according to the standard principle of real-time PCR primer designation (Shanghai Rui Jingsheng Biological Engineering Co., Ltd., Shanghai, China).

**Fluorescence real-time quantitative PCR detection.** The reaction mixture was as follows (50 μL total): 25 μL 2× PCR buffer, 0.6 μL×2 primers (25 pmol/μL), 0.3 μL SYBR green I (20×), 1 μL cDNA template, and 22.5 μL DEPC H₂O. The PCR amplification reaction conditions were as follows: 94 °C for 4 min; 94 °C for 20 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds for 35 cycles, following by 72 °C elongation. Dissolution curve analyses were performed on the amplified PCR products. Sequence Detection Software 2.2 was used to analyze the data and calculate the Ct value, which was the value for each sample to reach the threshold value during PCR amplification. The relative expression quantities of each sample (Bcl-2/B-actin, Bax/B-actin, caspase-3/B-actin, and Aβ/B-actin) were obtained following B-actin correction (Table 1).

**Western blot assay**

Following extraction of tissue protein, the protein concentration was determined using the BCA protein assay kit according to kit instructions. The OD562 value and the protein concentration standard curve were used to calculate the total sample protein concentration. Tissue extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Equal amounts of protein were subjected to electrophoresis on 10% SDS-PAGE gels and then the proteins were

---

**Table 1 Polymerase chain reaction primers**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Amplified fragment length</th>
<th>bp</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Upstream</td>
<td>GTGAGCTGGGGGAGGATTGT</td>
<td>167</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>GCATCCCGACCTCCGTTTA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bax</td>
<td>Upstream</td>
<td>CCCGAGAGGTCTCTCTCCCG</td>
<td>167</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>GAAGTTCCAGTGCAGCCCA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Upstream</td>
<td>CGAAGCTTTCTCATCACGAGGC</td>
<td>129</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>AGTAAGCATACAGGAAGTCGCC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Aβ</td>
<td>Upstream</td>
<td>CTGGAGGTGCCAACGATG</td>
<td>150</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>GGGTCTGACTCCCATTTC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-actin</td>
<td>Upstream</td>
<td>CCCATCTATGAGGTTAAGC</td>
<td>150</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Downstream</td>
<td>TTAATGTCAAGGACAGTTTC</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
transferred to polyvinylidene difluoride (PVDF) membranes using an electrophoretic transfer system. The PDVF membranes were then blocked for 1 h at room temperature and incubated with primary antibodies (Bax, Bcl-2, and caspase-3 were diluted 1: 500, and Aβ was diluted 1: 100 in TBS) overnight at 4 °C. After three washes with TBST for 5 min each, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1: 1000) at room temperature for 2 h. Finally, after washing the membranes three times with TBS for 5 min each, immune-labeled bands were identified by using a chemiluminescence-based detection kit (Pierce). The OD values for Bax, Bcl-2, caspase-3, Aβ, and β-actin were obtained using the Quantity One Gel Analysis System (Bio-Rad, Hercules, USA) as follows: target-protein optical density ratio = optical density value of the target protein. To reduce experimental error, the western blot assay was performed three times for each rat.

**Immunohistochemistry and quantitative analysis**

The fixed brain tissues were dehydrated through an alcohol gradient, followed by xylene. The tissues were then embedded in paraffin and sectioned on a cryostat at a thickness of 5 μm. Sections were dewaxed and subjected to 3% H2O2 for 10 min, followed by several washes in distilled water. Then sections to heat-mediated antigen retrieval with 0.01 M citric acid buffer (pH 6.0). Following several washes in phosphate-buffered saline (PBS), sections were blocked with 10% goat serum (30 min), and then incubated with rabbit anti-rat BAX antibody (1: 1000), Bcl-2 antibody (1: 1000), Caspase-3 antibody (1: 1000) and Aβ antibody (1: 1000) at 4 °C overnight. After incubation with the goat secondary antibody, at room temperature for 2 h. After detected with a DAB staining kit, the sections were counterstained with hematoxylin. The MIAAS Medical System (Media Cybernetics, Rockville, MD, USA) was used for image analysis. A total of six sections were selected for each group, and five fields were analyzed on each section (× 400 magnification). All positive cells were selected within the view, and the computer software IPP6.0 image analysis system (Media Cybernetics) automatically calculated the cell density. The positive target area/total area of the field of view was calculated for quantitative expression.

**TUNEL assay**

To detect cells undergoing apoptosis, TUNEL was performed according to the manufacturer’s protocol supplied within the TUNEL-pod kit (Roche, Basle, Switzerland). The brain sections were first immersed in xylene and dehydrated through serial alcohol dilutions followed by a wash step in distilled water. After treating with 3% H2O2 for 10 min at room temperature, the sections were incubated with proteinase K for 20 min at 37 °C to enhance permeability. Then, the sections were incubated for 60 min with TUNEL re-
Comparison of Bcl-2, Bax, caspase-3, and Aβ protein expressions in the cortex of AD rats

To investigate changes in Bcl-2, Bax, caspase-3, and Aβ protein expression in the cortex, we used western blot analysis. Results are shown in Figure 2. There was no significant different in Bcl-2, Bax, caspase-3, and Aβ protein expressions between the normal control group and the surgery control group (P > 0.05). Bax, caspase-3, and Aβ protein expression significantly increased in the model control group rats, and Bcl-2 protein significantly decreased, compared with the sham-surgery group (P > 0.01). Compared with the model control group, Bcl-2 mRNA expression in each treatment group significantly increased, but Bax, caspase-3, and Aβ mRNA expressions significantly decreased (P < 0.01). However, there were no differences in Bcl-2, Bax, caspase-3, and Aβ protein expression between the middle-dose QK group and the Aricept group (P > 0.05) (Figure 2).

Immunohistochemistry of Bcl-2, Bax, caspase-3, and Aβ in the cortex

In the AD model group, there was a significant increase in the number of cells expressing Bax, caspase-3, and Aβ in the cortex, and a significant decrease in the number of cells expressing Bcl-2 (P < 0.01; Figure 3) compared with the other groups. Following treatment with donepezil or QKF, these results were reversed. The 9.5 mg·kg⁻¹·d⁻¹ QK dose produced the strongest effect, which was compared with 4.75 and 19 mg·kg⁻¹·d⁻¹.

TUNEL assay results

Microscopic inspection of the cortical sections from normal control and sham-surgery rats revealed morphologically normal neurons with no TUNEL reaction. Compared with the control group, the number of apoptotic cells significantly increased in the model group (P < 0.01; Figure 4). After treatment with donepezil or QK, the number of TUNEL-positive cells significantly decreased compared with the model group (P < 0.01). The 9.5 mg·kg⁻¹·d⁻¹ QK dose produced the strongest effect, which was comparable to 4.75 or 19 mg·kg⁻¹·d⁻¹.

HE staining

HE staining revealed no remarkable neuronal abnormalities in the cortex of rats in the control and sham-surgery groups. The pyramidal cells were neatly and tightly arranged, and no cell loss was found. Additionally, for these groups, the cells were round and intact with clear, dark-blue nuclei (Figure 5). However, obvious cortical histopathological damage was observed in the model groups. The pyramidal layered structure was disintegrated, and neuronal loss was found. Neurons with pyknotic nuclei and with a shrunken or irregular shape were also observed (Figure 5C). These abnormalities were attenuated by treatment. The cells in the Aricept and QK groups exhibited better cell morphology and were more numerous than in the model group, but were overall worse than in the control and sham-surgery groups (Figure 5D, 5E).

DISCUSSION

AD is the most common type of dementia. The incidence of AD increases with age, which is compounded...
by the fact that people are living longer. There are many hypotheses for AD etiology, including the Aβ waterfall hypothesis,4,5 immune and inflammatory involvement hypothesis,6-11 the cholinergic defects hypothesis,12,13 the tau protein hyperphosphorylation hypothesis,14,15 the intracellular calcium homeostasis disorders hypothesis,14,16 and the peroxidation hypothesis.17 It has been suggested that the incidence of AD is caused by multiple factors, and Aβ deposition in neuronal cells of the brain is the initial event that occurs in AD.18 Therefore, for the present study, the bilateral hippocampi of rats were injected with Aβ1-40 fragments to establish an experimental model of AD. This method has been shown to be stable and reliable and very effectively simulates the pathological and pathophysiological characteristics of AD.19,22

Aβ is neurotoxic and induces apoptosis through a series of pathological and physiological mechanisms that lead to AD,23 such as activation of glial cells, which initiates neuroinflammation,24 induction of the inflammatory cascade,25 induction of oxidative stress mechanisms,26,27 and excessive expression of NO and NO toxicity.28 Taken together, these results suggest that Aβ-induced neuronal apoptosis is an important pathological characteristic of AD. Caspase-3 is an effector of apoptosis and the final executor of apoptosis.29 The Bcl-2 family is intricately involved in neuronal apoptosis; Bcl-2 is the most important pro-apoptotic gene in this family,30 and the ratio between these two genes plays a role in the physiological state. Previous experiments31 have shown that Aβ can lead to increase toxicity in neural stem cells, reduced Bcl-2 expression, increased Bax expression, and imbalanced Bax/Bcl-2 ratios, all of which undermine the integrity of cell membranes. Compared with the model group, Bcl-2 expression increased and Bax, caspase-3, and Aβ expression decreased in the cortex of each QK treatment group. In conclusion, QK significantly increased expression of Bcl-2, down-regulated expression of Bax, caspase-3, and Aβ, and reduced the number of apoptotic cells in the cortex.

REFERENCES
5. Hu HY, Cui ZH, Li HQ, et al. Fumanjian, a Classic Chi-
Figure 3 Immunohistochemical staining of the cortex (×400)
A1−H1: OD level of BAX in all groups. A2−H2: OD level of Bcl-2 in all groups. A3−H3: OD level of caspase-3 in all groups. A4−H4: OD level of Bcl in all groups. A: control; B: sham-surgery; C: control model; D: Aricept; E: low-dose QK; F: middle-dose QK; G: high-dose QK. 0+NS: normal control group (no treatment); NS+NS: sham-surgery group (no treatment); AB+NS: model control group (no treatment); Aricept: treated with Aricept (1.67 mg·kg⁻¹·d⁻¹); L-FJ: treated with QK (low dose of 0.15 mg·kg⁻¹·d⁻¹); M-FJ: treated with QK (medium dose of 0.5 mg·kg⁻¹·d⁻¹); H-FJ: treated with QK (high dose of 1.5 mg·kg⁻¹·d⁻¹). NS: normal saline; AB: amyloid-β protein; L-FJ: low dose of QK (4.75 mg·kg⁻¹·d⁻¹); M-FJ: medium dose of QK (9.5 mg·kg⁻¹·d⁻¹); H-FJ: high dose of QK (19 mg·kg⁻¹·d⁻¹). Data are presented as mean ± standard deviation (n = 6). Significant differences compared with NS + NS (sham-surgery) group are designated as *P < 0.05 and with AB + NS (model control) group as **P < 0.01 and ***P < 0.001.
A: control; B: sham-surgery; C: control model; D: Aricept; E: low-dose QK; F: middle-dose QK; G: high-dose QK. Apoptosis is expressed as the percentage of the number of TUNEL-positive cells to the total number of cells. 0+NS: normal control group (no treatment); NS+NS: sham-surgery group (no treatment); Aβ+NS: model control group (no treatment); Aricept: treated with Aricept (1.67 mg·kg⁻¹·d⁻¹); L-FJ: treated with QK (low dose of 4.75 mg·kg⁻¹·d⁻¹); M-FJ: treated with QK (medium dose of 9.5 mg·kg⁻¹·d⁻¹); H-FJ: treated with QK (high dose of 19 mg·kg⁻¹·d⁻¹). NS: normal saline; Aβ: amyloid-β protein; L-FJ: low dose of QK (4.75 mg·kg⁻¹·d⁻¹); M-FJ: medium dose of QK (9.5 mg·kg⁻¹·d⁻¹); H-FJ: high dose of QK (19 mg/kg/d). TUNEL: Terminal-deoxynucleotidyl Transferase mediated nick end labeling. Data are presented as mean ± standard deviation (n = 6). Significant differences compared with NS + NS (sham-surgery) group are designated as ‘$P < 0.01$ and with Aβ + NS (model control) group as ‘$P < 0.01$ and ‘$P < 0.001$. 

Figure 5 HE staining showing cellular morphology in the cortex (× 200) 
A: control (no treatment); B: sham-surgery (no treatment); C: control model (no treatment); D: Aricept (1.67 mg·kg⁻¹·d⁻¹); E: QK groups (medium dose of 9.5 mg·kg⁻¹·d⁻¹). Rats in the control and sham-surgery groups did not show histopathological abnormalities. In the model group, remnants of the pyramidal cells were irregularly arranged and some exhibited a shrunken and irregular shape. Cells in the Aricept and QK groups exhibited better cell morphology and were more numerous than in the model group. HE: hematoxylin eosin.


