Sericin can reduce hippocampal neuronal apoptosis by activating the Akt signal transduction pathway in a rat model of diabetes mellitus**☆

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Abstract
In the present study, a rat model of type 2 diabetes mellitus was established by continuous peritoneal injection of streptozotocin. Following intragastric perfusion of sericin for 35 days, blood glucose levels significantly reduced, neuronal apoptosis in the hippocampal CA1 region decreased, hippocampal phosphorylated Akt and nuclear factor kappa B expression were enhanced, but Bcl-xL/Bcl-2 associated death promoter expression decreased. Results demonstrated that sericin can reduce hippocampal neuronal apoptosis in a rat model of diabetes mellitus by regulating abnormal changes in the Akt signal transduction pathway.

Key Words: sericin; type 2 diabetes mellitus; hippocampus; apoptosis; Akt signal transduction pathway; neural regeneration

INTRODUCTION

One floss from the cocoon shell of the Bombyx mori silkworm, a member of the Bombycidae family¹, is composed of two parallel monofilament fibers. Each monofilament has a silk protein center that is surrounded by sericin. To date, most studies have focused on the application of sericin protein in cosmetology, skin care, nutrition, as an anti-oxidant and as a potential anticancer therapy²-³. Our previous studies have demonstrated that sericin can effectively protect islet cells, the testis and the kidney from injury in diabetic rats⁴-⁶. The protective effects of sericin on nervous system lesions in diabetes mellitus remain poorly understood, however, sericin has exhibited neuroprotective effects in a rat model of diabetes mellitus by inhibiting cortical and hippocampal heme oxygenase1 expression⁷. The hippocampus is sensitive to chronic hyperglycemia in diabetes mellitus, which manifests as a reduction in hippocampal neuron number, abnormal morphology, learning and cognitive dysfunction, delayed responses to surroundings, and cognitive impairment characterized by acquired cognitive and behavioral deficiency⁸-⁹. However, in rats with diabetes mellitus and cognitive impairment, the number of apoptotic neurons in the hippocampal CA1 region was significantly increased, indicating neuronal apoptosis participates in hippocampal injury-induced cognitive impairment following diabetes mellitus¹⁰. The Akt signal transduction pathway is composed of Akt, nuclear factor kappa B (NF-κB) and the Bcl-xL/Bcl-2 associated death promoter (Bad), and is involved in cell proliferation, apoptosis, differentiation and metabolism¹¹-¹². Akt is extensively expressed in tissues, and phosphorylated Akt (p-Akt) plays a role in cell metabolism, survival, proliferation, apoptosis, cell cycle regulation, and angiogenesis¹³-¹⁵. p-Akt can phosphorylate the inhibitory protein of NF-κB (IκB) by activating the inhibitory protein of NF-κB kinase to dissociate NF-κB from NF-κB-IκB. This restores NF-κB activity, and inhibits nerve cell apoptosis¹⁶-¹⁷. In addition, p-Akt phosphorylates Bad. Activated Bad can bind to the chaperonin protein 14-3-3 to block dimer formation of Bad and Bcl-2/or Bcl-xL¹⁸-¹⁹. Therefore, the activated Akt signal transduction pathway can relieve hippocampal neuronal injury²⁰. The present study investigated the effects of sericin on the hippocampal Akt signal transduction pathway in a rat model of type 2 diabetes mellitus to confirm the protective effects of sericin on hippocampal injury in type 2 diabetes mellitus.

RESULTS

Quantitative analysis of experimental animals
A total of 30 adult Sprague-Dawley rats were
used. Ten were selected for the control group, and the remaining 20 were used to establish a model of type 2 diabetes mellitus, and subdivided into model (no treatment) and sericin (sericin perfusion) groups. All 30 rats were included in the final analysis.

**Sericin reduced blood glucose levels in rats**
The blood glucose levels in model rats were significantly reduced following sericin treatment for 35 days ($P < 0.01$; Table 1).

**Sericin inhibited neuronal apoptosis in the hippocampal CA1 region of rats**
Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) detection showed neuronal apoptosis in the hippocampal CA1 region of each group. The number of apoptotic neurons was significantly elevated in model rats when compared with the control group ($P < 0.01$). Following sericin treatment for 35 days, the number of apoptotic neurons significantly decreased ($P < 0.05$; Table 1, Figure 1).

**Sericin promoted p-Akt, NF-κB protein and mRNA expression, but inhibited Bad protein and mRNA expression in the hippocampus of rats**
Protein and mRNA levels of p-Akt, NF-κB and Bad in the hippocampus were detected using western blotting and reverse transcription (RT)-PCR. p-Akt and NF-κB protein and mRNA expression was significantly reduced in the hippocampus of rats with diabetes mellitus ($P < 0.01$), while Bad protein and mRNA levels were significantly elevated ($P < 0.01$). Following sericin treatment for 35 days, p-Akt and NF-κB protein and mRNA expression was significantly increased ($P < 0.01$), but Bad protein and mRNA levels were significantly decreased in the hippocampus ($P < 0.01$; Figure 2, Table 2).

![Figure 1](image1.jpg)

**Neuronal apoptosis in the hippocampus (TUNEL, × 200).**
Apoptotic neurons had brown/yellow stained nuclei. Compared with the control group (A), the number of apoptotic neurons in the hippocampal CA1 region was significantly increased in the model group (B), but the sericin group (C) exhibited significantly less apoptotic neurons in the hippocampal CA1 region. TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

![Figure 2](image2.jpg)

**Protein and mRNA levels of phosphorylated Akt (p-Akt; A), nuclear factor kappa B (NF-κB; B) and Bcl-xL/Bcl-2 associated death promoter (Bad; C) expression in the hippocampus using western blotting and reverse transcription-PCR.** p-Akt and NF-κB protein and mRNA expression was significantly increased following sericin treatment for 35 days. Bad protein and mRNA expression was significantly decreased following sericin treatment for 35 days.

1: Control group; 2: model group; 3: sericin group; β-actin: internal reference.

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**Table 1** Blood glucose levels and apoptotic index of neurons in the hippocampal CA1 region of rats in each group

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mM)</th>
<th>Apoptotic index of neurons in the hippocampal CA1 region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.12±2.22</td>
<td>0.088 ±0.008 6</td>
</tr>
<tr>
<td>Model</td>
<td>29.00±5.39* (13.874 7)</td>
<td>0.115 ±0.021 8* (5.630 1)</td>
</tr>
<tr>
<td>Sericin</td>
<td>14.03±3.98* (11.616 5)</td>
<td>0.100 ±0.013 5* (3.037 8)</td>
</tr>
</tbody>
</table>

*P < 0.01, vs. control group; $^*$P < 0.01, $^{**}$P < 0.05, vs. model group. The apoptotic index of neurons in the hippocampal CA1 region is represented as the ratio of the TUNEL-positive stained area to the total area of the field of view. Data were expressed as mean ± SD of ten rats in each group. Intergroup differences were compared using one-way analysis of variance, and paired comparison was performed using the q-test. ‘( )’ represents q value.
DISCUSSION

In the present study, the hippocampal Akt signal transduction pathway exhibited abnormal changes in rats with diabetes mellitus, as manifested by decreased p-Akt and NF-κB expression and elevated Bad expression. Hyperglycemia in diabetes mellitus can inhibit Akt activation or accelerate Akt deactivation, and reduce Akt phosphorylation by activating the c-Jun N-terminal kinase pathway. Moreover, hyperglycemia in diabetes mellitus can induce hippocampal blood capillary injury, leading to regional ischemia and elevated Bad expression levels. In the present study, p-Akt and NF-κB expression was significantly elevated, but Bad expression was significantly reduced in the sericin group when compared with the model group. Interestingly, increased p-Akt expression has been shown to induce an anti-apoptotic effect, and phosphorylate and activate NF-κB and Bad. Therefore, results from the present study indicate that sericin can reduce hippocampal neuronal apoptosis and protect the hippocampus against injury by regulating the Akt signal transduction pathway in rats with diabetes mellitus. Moreover, sericin is a natural protein and will reduce the possibility of toxic side effects that can occur from chemically synthesized drugs. However, the mechanism underlying how sericin regulates the Akt signal transduction pathway needs further investigation.

MATERIALS AND METHODS

Design
A randomized, controlled, animal study.

Time and setting
The experiment was performed at the Institute of Basic Medicine, Chengde Medical College, China, from June 2009 to October 2010.

Materials
Animals
A total of 30 healthy, male, Sprague-Dawley rats, aged 3 months, weighing 200–250 g, were provided by the Laboratory Animal Center of Hebei Medical University (No. 712024). They were housed at 20 ± 2°C in 40–70% humidity. The experimental procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China.

Drugs
Sericin was made from the colored silk worm cocoon (Sericultural Research Institute, Chengde Medical College) and was prepared by soaking, decocting, filtrating and condensing, according to a previously described method.

Methods
Establishment of type 2 diabetes mellitus and sericin administration
The rat model of diabetes mellitus was established by intraperitoneal injection of 2% (w/v) streptozotocin (25 mg/kg; Sigma, St. Louis, MO, USA) for 3 consecutive days. A successful model was demonstrated when rats had blood glucose levels ≥16.7 mM. Sericin group rats were intragastrically perfused with sericin, 2.4 g/kg per day immediately following model establishment for 35 consecutive days.

Blood glucose detection
Blood glucose levels in the model group were detected following model establishment, and were detected 35 days following sericin administration in the sericin group. The rats were fasted for 12 hours or more, and anesthetized by intraperitoneal injection of 4% (w/v) chloral hydrate. Blood (about 3 mL) was collected from the medial angle of the eye, and centrifuged at 3000 r/min for 20 minutes. The supernatant was collected, and blood glucose levels was detected using the glucose oxidase method, glucose detection kit (Boaoing Great Wall Clinical Reagents Co., Ltd., Baoding, Hebei Province, China; No. 20071030) and Boehringer Mannheim/Hitachi 717 automatic clinical biochemical analyzer (Hitachi, Tokyo, Japan).

Preparation of brain tissue samples
The brain was harvested after rats were anesthetized and sacrificed. The hippocampus from one hemisphere was rapidly isolated on ice and stored in liquid nitrogen, and the hippocampus from the remaining hemisphere was fixed in Bouins solution (75 mL saturated picric acid

Table 2 Hippocampal phosphorylated Akt (p-Akt), nuclear factor kappa B (NF-κB) and Bcl-xL/Bcl-2 associated death promoter (Bad) expression (absorbance ratio of target band to β-actin)

<table>
<thead>
<tr>
<th>Group</th>
<th>p-Akt Protein</th>
<th>p-Akt mRNA</th>
<th>NF-κB Protein</th>
<th>NF-κB mRNA</th>
<th>Bad Protein</th>
<th>Bad mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.278±0.036</td>
<td>0.692±0.017</td>
<td>0.584±0.024</td>
<td>0.298±0.018</td>
<td>1.309±0.021</td>
<td>0.338±0.014</td>
</tr>
<tr>
<td>Model</td>
<td>0.372±0.026*</td>
<td>0.224±0.014*</td>
<td>0.294±0.024</td>
<td>0.134±0.022*</td>
<td>0.341±0.012*</td>
<td>0.948±0.025*</td>
</tr>
<tr>
<td>Sericin</td>
<td>0.984±0.036#</td>
<td>0.460±0.027*</td>
<td>0.493±0.012*</td>
<td>0.687±0.031*</td>
<td>0.528±0.018* (68.531)</td>
<td>0.542±0.013# (113.672)</td>
</tr>
</tbody>
</table>

*P < 0.01, vs. control group; #P < 0.01, vs. model group. Data were expressed as mean ± SD of ten rats in each group. Intergroup differences were compared using one-way analysis of variance, and paired comparison was performed using the q-test. (*) represents q value.
The hippocampus stored in liquid nitrogen was collected from each rat, and 10 rats from each group were used.

Western blotting for hippocampal p-Akt, NF-κB and Bad protein expression

The hippocampus (100 mg) stored in liquid nitrogen was placed in 1 mL pre-cooled phosphate buffered saline (0.01 mM, pH 7.4), homogenized on ice, centrifuged, and the supernatant was discarded. The sediments were treated with 400 µL of RIPA buffer (RIPA: PMSF = 100:1), left on ice for 40 minutes, and centrifuged at 12 000 r/min at 4°C for 15 minutes. The supernatant was retained. The concentration of protein was quantified using the BCA protein kit (Beijing Taigemei Science and Technology, Beijing, China)[30]. Samples were electrophoresed on a 15% (w/v) SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were incubated with rabbit anti-p-Akt polyclonal antibody (1: 100; Beijing Biosynthesis Biotechnology, Beijing, China), mouse anti-NF-κB (1: 100), rabbit anti-Bad (1: 200) polyclonal antibody (Beijing Biosynthesis Biotechnology) and mouse anti-rat β-actin monoclonal antibody (1: 1 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by goat anti-rabbit or mouse IgG (1: 5 000; KPL, Gaithersburg, Maryland, USA) at room temperature for 1 hour. Development was performed using Super ECL Plus enhanced chemiluminescence (Beijing Taigemei Science and Technology, Beijing, China). The film was scanned using an EPSON scanner (Epson, Beijing, China), and developed bands were analyzed using Quantity One-4.6.2 software (Bio-Rad, Hercules, CA, USA). Protein expression levels were determined as the absorbance ratio of the target band to β-actin.

RT-PCR detection for hippocampal p-Akt, NF-κB and Bad mRNA expression

The hippocampus stored in liquid nitrogen was collected and total RNA was extracted according to the Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed into cDNA. PCR primers were synthesized by Sangon, Shanghai, China. PCR primer sequence and amplification conditions:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>TA (°C)</th>
<th>Cycle</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt</td>
<td>F: 5′- AGA TCC TCA AGA AAG AAG TCA TC-3′&lt;br&gt;R: 5′- TTG TTC TCG GAG TGC AAG TAG TC-3′</td>
<td>60.1</td>
<td>32</td>
<td>271</td>
</tr>
<tr>
<td>NF-κB</td>
<td>F: 5′- TGC ATG ACC TTG CCA ACC-3′&lt;br&gt;R: 5′- AAA TTC TCC CCA AAC TCC ACC-3′</td>
<td>56</td>
<td>31</td>
<td>380</td>
</tr>
<tr>
<td>Bad</td>
<td>F: 5′- GGC GCT TTG TCG CAT CTG TG-3′&lt;br&gt;R: 5′- TCC TTC TCC TTT GGA GCT TCC-3′</td>
<td>60</td>
<td>31</td>
<td>396</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5′- GAG GGA AAT CGT GCG TGA C-3′&lt;br&gt;R: 5′- CTG GAA GGT GGA CAG TGA G-3′</td>
<td>55</td>
<td>29</td>
<td>445</td>
</tr>
</tbody>
</table>

PCR conditions: 94°C for 2 minutes; 94°C for 30 seconds; 50−65°C for 30 seconds; 72°C for 1 minute, circulated from the second step. Amplified products, 5 µL and DNA Ladder, 5 µL (Beijing Taigemei Science and Technology) were used for 2% (w/v) agarose gel electrophoresis at 90 V for 40 minutes. Images were photographed using the ZF UV transmittance analyzer (Shanghai Jiapeng, Shanghai, China) and quantitatively analyzed using Quantity One-4.6.2 software. Target gene mRNA expression levels were represented by the absorbance ratio of the target band to β-actin[31].

Statistical analysis

Data were expressed as the mean ± SD and analyzed using SPSS 11.5 software (SPSS, Chicago, IL, USA). Intergroup differences were compared using one-way analysis of variance, and paired comparison was performed using the q-test. A value of P < 0.05 was considered statistically significant.

Author contributions: Zhihong Chen was in charge of funds, conceived and designed the study, analyzed data and wrote the manuscript. Yaqi He analyzed the data. Chengjun Song fed the animals and provided experimental data. Zhijun Dong and Zhejun Su conducted the statistical analysis. Jingfeng Xue conceivcd and designed the study, revised the manuscript and guided the study.

Conflicts of interest: None declared.

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Ethical approval: This study received permission from the Animal Ethics Committee of Hebei Province, China.

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REFERENCES


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