Anti-Apoptosis Effect of Astragaloside Iv on Alzheimer's Disease Rat Model via Enhancing the Expression of Bcl-2 And Bcl-Xl

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Summary
The aim is to explore the protective effect of Astragaloside IV on Alzheimer’s disease (AD) in rats induced by amyloid-ß peptide (Aβ₁₋₄₂) and its potential therapeutic mechanism. Methods: 50 Male Sprague Dawley rats were divided into five groups (10 rats for each): control group, model group, treatment groups 1~3. 10µg Aβ₁₋₄₂ was injected bilaterally into the dorsal dentate gyrus of the hippocampus of rats in the model and treatment groups to prepare the AD models. 24h after modeling, Astragaloside IV administration, with different drug dosages of 20mg/(kg•day), 40mg/(kg•day) and 60mg/(kg•day), was performed by gastric perfusion for rats in the treatment group 1~3. Later on, the cognitive ability of rats was examined by a series of behavioral tests, and the expression of Bcl-2 and Bcl-xl in the hippocampus of rats was detected by the fluorescein based Quantitative RT-PCR. Results: The spontaneous alternation test in a Y maze and Morris water maze task have demonstrated that the repeated daily administration of Astragaloside IV at the doses of 20mg/kg bw/day) (p<0.05), 40mg/kg bw/day) (p<0.01), and 60mg/kg bw/day) (p<0.01) significantly ameliorated the impairment of performance caused by Aβ₁₋₄₂. Furthermore, Astragaloside IV also enhanced the expression of Bcl-2 and Bcl-xl in hippocampal neurons of rats in a dosage-dependent manner. Conclusion: These findings suggest that Astragaloside IV could alleviate cognitive impairment and enhance the expression of Bcl-2 and Bcl-xl in hippocampus of rat models with AD.

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Introduction
Alzheimer’s disease (AD), a neurodegenerative disease, is affecting more than 25 million people worldwide (Xiongwei Zhu et al., 2007). The decline of memory and cognition in AD patients is underlain by untimely synaptic loss, enhanced inflammatory signaling, the progressive deposition of senile plaques and neurofibrillary tangles, and neuronal degeneration (Arias E et al, 2005). The accumulation of extracellular amyloid-ß (Aß) peptide derived from the transmembrane glycoprotein ß-amyloid precursor protein (ßAPP) in the limbic system via ß-γ-secretase cleavage is characteristic of AD (Carl Nathan et al., 2005). In addition, apoptosis has been demonstrated to play an important role not only in neuronal development and differentiation of the central nervous system (CNS) but also in the pathogenesis of AD. Bcl-2 and Bcl-xl, as the anti-apoptotic proteins, are regulated by the mitochondrial pathway which is now widely considered to be an important step in controlling and initiating apoptosis in AD (Drache B et al., 1997). In the past two decades, many efforts have been made to understand the molecular pathogenesis of AD, and to carry out its early diagnosis and therapeutic control.

Contemporary research has proven that many tra-
ditional Chinese herbal extracts and isolated compounds possess some potential medical effectives, interest in which has boomed in the western countries. It is very important to study their molecular mechanisms and purify effective compounds with new knowledge and new techniques to meet a great need for human health. Among them, the dried root of *Astragalus membranaceus* (Huangqi) has a long history of medicinal use. It is now commonly used as an immunomodulating agent in mixed herbal decoctions to treat common cold, diarrhea, fatigue and anorexia, and is being prescribed to patients with cardiac diseases (Xiao-Dong CHENG et al., 2004; Normile D et al., 2003). In recent years, Astragaloside IV has also been used as a kind of anti-ageing drug (Cai Q et al., 2001). It has been demonstrated that Astragaloside IV has a significant mitogenic activity as well as the ability to improve the metabolism and survival time of cells. It was found by Chinese scientists in the 1970s to have the potential to increase the cumulative population doubling of human fetal lung diploid fibroblasts and human fetal kidney cells *in vitro*, furthermore, the delaying effect of the crude drug on ageing was initially identified in rats (Wang XJ et al., 2001).

Based on the above observations, we have investigated the protective effect of Astragaloside IV on AD rats induced by Aβ1-42 and its potential therapeutic mechanism, in order to develop AD therapeutics and extend the use of Chinese Medicine all over the world.

**Materials and Methods**

**Animals**

Adult male Sprague-Dawley (SD) rats (*n*=50, 220~250g, Experimental Animal Center of University) were housed 5 per cage with free access to food and water, and were kept in a constant environment (22 ± 2°C, 50 ± 5% humidity, 12h light/dark cycle). All experimental animals were overseen and approved by the Animal Care and Use Committee of Changzheng Hospital of Chinese Second Military Medical University before and during experiments.

**Chemicals and reagents**

Astragaloside IV was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Shanghai, PR. China (110781-200613). Aβ<sub>1-42</sub> was purchased from Sigma, St. Louis, MO, USA.

All chemicals were of analytical reagent grade. Before the experiment, all of the vessels and tips for pipetting were dipped in strong HNO<sub>3</sub> for 24 h and then washed with ultrapure water. The water used was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

**Preparation of Aβ<sub>1-42</sub>-Infused Alzheimer’s Disease Rat Model**

Aβ<sub>1-42</sub> was dissolved in sterile distilled water at a concentration of 5µg/µL, and incubated at 37°C for 7 days to obtain the aggregated form. Under anesthesia, a microinjection of Aβ<sub>1-42</sub> (1µL=5µg) was performed into the bilateral hippocampuses using stereotaxic coordinates derived from the atlas of Paxinos and Watson (Paxinos G et al., 1986). All microinjections were made using a 10 µl Hamilton syringe, with 26-gauge stainless steel needle, lowered slowly into place. The needle was left in place for 3-5 min before the injection was started, and then the fragments were injected slowly at a rate of 0.1µl/min. The needle was left in place for an additional 3-5 min before being slowly withdrawn. As a control, in the same operating session and using a different microsyringe, a single microinjection of identical volume of vehicle solution (1 µl of PBS) at a rate of 0.1 µl/min was made into the corresponding regions of hippocampuses using the same stereotactic coordinates as for the Aβ<sub>1-42</sub> injection. After the injection, the scalp was sutured and the rats were allowed to recover from the anaesthetic.

**Grouping and drug treatment**

50 Male SD rats were randomly divided into five groups (10 rats for each): control group, model group and treatment groups1~3. Experimental dosages of Astragaloside IV were prepared in distilled drinking water (5mg/100ml, 10mg/100ml and
15mg/100ml water). 24 h after Aβ1-42 injection, rats in the treatment groups 1~3 began to receive Astragaloside IV administration by gastric perfusion (target dose: 20mg/kg bw/day, 40mg/kg bw/day and 60mg/kg bw/day), continuously for 5 days, once per day. No medication was administered in the model group and the control group.

Behavioral testing
Spontaneous alternation test (in Y-maze)
2 days after drug administration, the function of the rat hippocampus was evaluated by recording spontaneous alternation behavior during an 8-min session in a Y-maze (Yamada K et al., 1996). During this test, rats tended to explore the maze systematically, entering each arm in turn. The sequence of arm entries was recorded manually. An actual alternation was defined as entries into all three arms on consecutive occasions. Therefore, the number of maximal alternation was the total number of arm entries minus 2, and the percentage of alternation was calculated as (actual alternations/maximal alternations)×100. In addition, the total number of arms entered during the sessions was also determined.

Place learning (in water maze)
The function of the rat hippocampus was also evaluated by the Morris water maze (Morris R., 1984), which was conducted on the day following the Y-maze test. The experimental apparatus consisted of a black circular water tank (140 cm in diameter and 45 cm high). A transparent platform (10 cm in diameter and 25 cm high), which could not be seen by rats, was set inside the tank, which was filled to a height of 27 cm with water of temperature approximately 23°C; the surface of the platform was 2 cm below the surface of the water. The water tank was located in a test room, in which there were many cues external to the maze. The room had adjustable indirect illumination, and a camera was fixed to the ceiling. The position of the cues remained unchanged throughout the Water maze test.

Reference Memory Test
The reference memory test was carried out on five consecutive days and consisted of one trial per day. Each daily trial consisted of 5 training sessions with a 30 s gap between each session. For each training trial, the rat was put into the water tank at one of five starting positions; the same starting position was used for each session on the same day. The platform was located in a constant position throughout the test period in the middle of one quadrant, equidistant from the center and edge of the water tank. In each training session, the latency to escape onto the hidden platform was recorded. If a rat was unable to find the platform within 90 s, the rat was guided to the platform, and a maximal score of 90 s was assigned. The escape latency was analyzed using Target System (Neuroscience Inc., Tokyo, Japan). After each training session, the rat was allowed to remain on the platform for 15 s and then was returned to its home cage.

Probe Trial
Immediately after the 5th training trial, the platform was removed from the water tank, and animals were tested on a 30-s spatial probe trial. The time spent in the target quadrant, where the platform had been located during training, and the time spent in other quadrants were measured.

Fluorescein based quantitative RT-PCR assay
The expression levels of Bcl-2 and Bcl-xl in the hippocampus of rats in each group were detected by Fluorescein Based Quantitative RT-PCR. On the day following behavioral testing, the rats were sacrificed and perfused with 0.1 M phosphate-buffered saline (pH=7.4). The brain hemispheres (excluding cerebellum, pons, and medulla oblongata) were separated. Total RNA was extracted from the hippocampus tissues with a phenol/guanidine isothiocyanate based reagent (TrizolTM, Gibco BRL, Scotland). Briefly, 1 ml of Trizol and 200µl of chloroform were added to each tube. The RNA was then precipitated with 500µl of isopropanol (v/v) and washed with 75% ethanol and air dried. The purified RNA was then dissolved in 10 µl of diethyl pyrocarbonate (DEPC) treated water, the optical density measured at 260/280 wavelength and stored
at -80°C prior to use in the synthesis of cDNA. RNA was reverse transcribed using the Super scripte™ Preamplification System (Life Technology). 500ng of total RNA were mixed with 500ng of oligo (dT) primers and incubated at 70°C for 10 min. The mixture was then chilled on ice and incubated with a 1× reverse transcriptase buffer (50 mM Tris-HCl, pH 8.4, 75mM KCl), 3 mM MgCl₂, 500µM of each deoxynucleotide, 10mM dithiothreitol and 200U of Superscript II RT reverse transcriptase at 42°C for 50 min. The 20µl reactions were further incubated at 70°C for 15 min and 2 U RNase H added to each tube. Following a final incubation at 37°C for 20 min, the cDNAs were stored at -80°C until use.

PCR was performed in MJ Opticon Monitor 2.0 (MJ Ltd, USA) using SYBR Green I (Biogene) as fluorescein. The following pairs of primers were used: β-actin (258bp): 5’ GAC CTT CAA CAC CCC AGC CA 3’ (sense), 5’ GTC ACG CAC GAT TTC CCT CTC 3’ (antisense); Bcl-2 (385 bp): 5’ -ACT TGT GGC CCA GAT AGG CAC CCA G-3’ (sense), 5’ -ACT TTG GGC CCA GAT AGG CAC CCA G-3’ (antisense); Bcl-xl (448 bp): 5’-CCC AGA AAG GAT ACA GCT GG-3’ (sense), 5’-GCG ATC CGA CTC ACC AAT AC-3’ (antisense). All primers were synthesized by Sangon Co. (Shanghai, China). The PCR profile consisted of an initial melting step of 2 min at 94°C, followed by 39 cycles of 45 s at 94°C, 20 s at 62°C and 20 s at 72°C, and a final elongation step of 10 min at 62°C.

Sample quantification was carried out by the software of Sequence Detection System (SDS2.0) with constructing a standard calibration curve using serial dilutions of β-actin DNA of known concentration, from which the concentration of an unknown sample could be determined. In order to analyze inter-assay variation, a least square curve fitting test was used to construct a fit, and the standard error of the data points to the curve was estimated by plotting the log of the sample concentration against the number of cycles the PCR machine undertakes to yield a set of fluorescent data. The standard error was estimated to be 15% on the log and was within the values described for β-actin quantification using competitive PCR.

**Calculations and statistics**

Data obtained from above experiments were expressed as mean±S.E. and handled by SPSS12.0 software. For the results of Y-maze, probe trial of Morris water maze, data were analyzed using one-way analysis of variance (ANOVA), which was followed by the Dunnett’s multiple comparison test. For the

**Figure 1.** Effects of Astragaloside IV on spontaneous alternation behavior in Aβ₁₋₄₂-infused AD rats. Daily intragastric administration of Astragaloside IV was started on day 2 after modeling. Spontaneous alternation behavior (A) and the number of arm entries (B) during an 8-min session in the Y-maze test were measured on day 3 after Astragaloside IV administration. Vertical bars show mean±S.E. *, p<0.05 compared with the control group; **, p<0.01 compared with the control group; †, p<0.05 compared with the model group; ‡, p<0.01 compared with the model group.
results of reference memory test, data were analyzed by two-way ANOVA, which was followed by Tukey analysis. A difference between means was considered significant if the p value was less than 0.05.

**Results**

**Astragaloside IV prevented the alternation performance impairment in the AD rats in a spontaneous alternation test**

Spontaneous alternation is often considered as a test for exploration, because the visiting of novel arms in contrast to the just visited arm is believed to be driven by exploration. Therefore, decreased spontaneous alternation could be a result of a decreased tendency to explore. As shown in Figure 1A, there was a significant group effect on the spontaneous alternation behavior (p<0.01 by the one-way ANOVA). The one-way ANOVA analysis revealed that frequency of spontaneous alternation behavior in the Aβ_{1-42}-infused model group was significantly less than that in the control group (p<0.01 by the one-way ANOVA). Astragaloside IV at the doses of 20mg/kg (p<0.05), 40mg/kg (p<0.01 by the one-way ANOVA), and 60mg/kg (p<0.01 by the one-way ANOVA) significantly attenuated the impairment caused by Aβ_{1-42}. The number of arm entries did not differ among the five different treatment groups of rats (Figure 1B), indicating that changes in alternation behavior were not because of generalized exploratory, locomotor or motivational effects.

**Astragaloside IV prevented performance impairment in the AD rats in a place learning in water maze**

Changes in escape latency to find the hidden platform in training trials with rats of different groups are shown in Table 1. Two-way ANOVA with all of three treatment groups revealed significant main effects of group (p<0.01 by two-way ANOVA) and training (p<0.01) but no significant effect of group by trial interactions (p>0.05 by two-way ANOVA). Post hoc analysis indicated that performance in the Aβ_{1-42}-infused model group was significantly impaired compared with that in the control group. Repeated daily administration of Astragaloside IV at the doses of 20mg/kg (p<0.05 by two-way ANOVA), 40mg/kg (p<0.01 by two-way ANOVA), and 60mg/kg (p<0.01 by two-way ANOVA) significantly ameliorated the impairment of performance caused by Aβ_{1-42} (Table 1).

A 30-s spatial probe trial was carried out following the 5th training trial. One-way ANOVA indicated that there was a significant group effect on the time spent in the quadrant in which the platform had been located at the same place during training (p<0.05 by

<table>
<thead>
<tr>
<th>Groups (n=10)</th>
<th>Escape latency (s)</th>
<th>1&lt;sup&gt;st&lt;/sup&gt; trial</th>
<th>2&lt;sup&gt;nd&lt;/sup&gt; trial</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; trial</th>
<th>4&lt;sup&gt;th&lt;/sup&gt; trial</th>
<th>5&lt;sup&gt;th&lt;/sup&gt; trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71.15±17.00</td>
<td>60.82±13.67</td>
<td>51.09±11.45</td>
<td>46.79±12.88</td>
<td>35.31±9.72</td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>96.52±15.63**</td>
<td>86.13±12.61**</td>
<td>79.28±18.36**</td>
<td>72.59±17.19**</td>
<td>67.82±15.27**</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>20mg/kg</td>
<td>82.36±18.90***</td>
<td>79.65±18.33***</td>
<td>74.55±12.16***</td>
<td>68.72±11.71***</td>
<td>51.76±10.08***</td>
<td></td>
</tr>
<tr>
<td>40mg/kg</td>
<td>79.67±18.03#***</td>
<td>71.63±12.78#***</td>
<td>67.65±12.59#***</td>
<td>62.27±12.25#***</td>
<td>49.89±6.58#***</td>
<td></td>
</tr>
<tr>
<td>60mg/kg</td>
<td>73.29±19.11##</td>
<td>70.60±13.28##</td>
<td>63.30±14.11##</td>
<td>56.61±13.86##</td>
<td>44.79±12.41##</td>
<td></td>
</tr>
</tbody>
</table>

Note: *P<0.05 and **P<0.01 refers to the comparison with the control group. #P<0.05 and ##P<0.01 refers to the comparison with the model group.
Astragaloside IV at the doses of 20mg/kg ($p<0.05$ by the one-way ANOVA), 40mg/kg ($p<0.01$ by the one-way ANOVA), and 60mg/kg ($p<0.01$ by the one-way ANOVA) significantly attenuated the impairment caused by Aß$_{1–42}$ (Table 1).

Astragaloside IV increased the expression of Bcl-2 and Bcl-xl in hippocampus of AD rats

Astragaloside IV may protect cells from Aß$_{1–42}$-induced oxidative stress, and thereby reduce β-secretase activity and Aß-generation (Othman Ghribi, et al., 2001). Therefore, Bcl-2 and Bcl-xl, as neuroprotective proteins, which may be involved in the neuro-protective effects of Astragaloside IV, were measured in this study. Figure 2 and Table 2 shows that Astragaloside IV produced a significant change on the expression of Bcl-2 and Bcl-xl in the hippocampus tissues of rats in the different groups. A post hoc analysis indicated that Astragaloside IV at the doses of 20mg/kg ($p<0.05$ by post hoc analysis), 40mg/kg ($p<0.01$ by post hoc analysis), and 60mg/kg ($p<0.01$ by post hoc analysis) significantly increased the expression of the genes for Bcl-2 and Bcl-xl in the treatment groups ($p<0.05$ by post hoc analysis).

**Table 2.** Effect of Astragaloside IV on expression levels of Bcl-2 and Bcl-xl in hippocampus of AD rats ($\bar{X}$±S, ng/ml, n=10)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bcl-2</th>
<th>Bcl-xl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.07±1.80</td>
<td>10.29±1.67</td>
</tr>
<tr>
<td>Model</td>
<td>6.52±0.63**</td>
<td>4.13±0.51**</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
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<tr>
<td>20mg/kg</td>
<td>9.36±0.91***</td>
<td>6.65±0.83***</td>
</tr>
<tr>
<td>40mg/kg</td>
<td>11.67±0.43***</td>
<td>8.63±0.78***</td>
</tr>
<tr>
<td>60mg/kg</td>
<td>12.91±1.16##</td>
<td>9.68±1.05##</td>
</tr>
</tbody>
</table>

Note: * $p<0.05$, ** $p<0.01$ referred to comparison with the control group; # $p<0.05$, ## $p<0.01$ referred to comparison with the model group.

Discussion

AD is characterized by progressive cognitive dysfunction and the deposition of Aß, which is the event initiating the decades-long pathological cascade leading to the disease (LI LX et al., 2003). The present study showed that continuous infusion of Aß$_{1–42}$ into rat brain induced a significant disturbance in spontaneous behaviors in Y maze and Water maze tests, both of which are considered to involve spatial memory. Furthermore, Astragaloside IV administration significantly ameliorated impairment of spontaneous alternation behavior in the Y maze test, reference memory, and probe trial in the Water maze test at different dosages. The intragastric administration of Astragaloside IV at the doses of 60 mg/kg, but not 20 and 40 mg/kg, significantly shortened escape latency in Water maze test in Aß$_{1–42}$-infused rats. These results indicate that Astragaloside IV has an ameliorating effect on cognitive impairment caused by Aß$_{1–42}$.

Apoptosis is the process by which neurons die during normal development and is also a feature of
chronic and acute neurodegenerative diseases and stroke (Nicotera P et al., 2004). Many studies of apoptosis in AD have been performed on post-mortem tissue and the results will, of course, only reflect the end-stage situation of AD (Maria Ankarcrona et al., 2005; Guo Q et al., 1997). The identification of neuronal specific markers for cell death could be used as proof that the cells actually die by, for example, apoptosis and as a tool in the diagnostic work. It is very important to be able to give an early and correct diagnosis and a reliable marker for cell death could be of help. Such a marker could also be used in the evaluation of the effects of drugs preventing cell death. The Bcl-2 protein family consists of pro-apoptotic and antiapoptotic members that interact at both the physical and functional level to regulate mitochondrial integrity and apoptotic cell death (Kittamura Y et al., 1998). After activation, proapoptotic family members such as Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) target the mitochondria and cause membrane permeabilization and the release of pro-apoptotic effectors. Mitochondrial cytochrome c is considered to play a pivotal role in the initiation of apoptosis when released into the cytoplasm. On the other hand, members of the Bcl-2 family proteins are believed to determine cell life or death by inhibiting (as in the case of Bcl-2 and Bcl-xl), or promoting (as in the case of Bax), the release of cytochrome c (M. Saldan et al., 2007). For these reasons, the present study has been investigated the influence of Astragaloside IV on the expression level of Bcl-2 and Bcl-xl in the AD rat’s brain.

Astragaloside IV has been shown to have a protective effect on neurons against oxidative stress in cultured mesencephalic neurons and glial cells (Mandy M.Y.Tin et al., 2007), and to have anti-tumor properties in colon cancer cells and tumor xenograft (He Z.Q, 1991). The mechanisms by which Astragaloside IV exerts its neuroprotective effect are diverse, and recent evidence demonstrates that the rescue and repair of injured neurons is a consequence of an antiapoptotic action of Astragaloside IV. Indeed, Astragaloside IV has been shown to up-regulate Bcl-2 and Bcl-xl levels in rat mesencephalic neurons subjected to apoptosis, resulting in a reduction of caspase activation (Giannakopoulos P et al., 1999). In the present investigation, we have demonstrated that treatment with Astragaloside IV at doses of 20, 40, and 60 mg/kg enhanced the expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-xl, and in a dose-related manner, thus dramatically inhibiting apoptosis.

In summary, intragastric administration of Astragaloside IV ameliorates impairment of hippocampal function and up-regulates the expression of Bcl-2 and Bcl-xl to inhibit apoptosis of neurons. For the first time, these findings suggest that Astragaloside IV might alleviate cognitive impairment and inhibit neuronal death associated with AD.

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