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Radix Achyranthis Bidentatae improves learning and memory capabilities in ovariectomized rats☆

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Abstract

Kidney-tonifying recipe can reduce the accumulation of advanced glycation end products, prevent neuronal degeneration and improve cognitive functions in ovariectomized rats. Radix Achyranthis Bidentatae alcohol extracts may dose-dependently inhibit non-enzymatic saccharification *in vitro*. This study aimed to examine the effect of Radix Achyranthis Bidentatae on advanced glycation end products and on learning and memory capabilities in ovariectomized rats. Ovariectomized rats were treated with Radix Achyranthis Bidentatae alcohol extracts (containing 1.5 g/kg crude drug) or 0.1% aminoguanidine for 12 weeks and behavioral testing was performed with the Y-electrical maze. This test revealed that Radix Achyranthis Bidentatae and aminoguanidine could improve the learning and memory capabilities of ovariectomized rats. Results of competitive enzyme-linked immunosorbent assay showed that treatment with Radix Achyranthis Bidentatae or aminoguanidine reduced the accumulation of advanced glycation end products in the frontal cortex of ovariectomized rats, while increasing content in the blood and urine. Biochemical tests showed that treatment with Radix Achyranthis Bidentatae or aminoguanidine decreased superoxide dismutase activity in the serum and frontal cortex, and increased serum levels of glutathione peroxidase in ovariectomized rats. In addition, there was no apparent effect on malondialdehyde levels. These experimental findings indicate that Radix Achyranthis Bidentatae inhibits production of advanced glycation end products and its accumulation in brain tissue, and improves learning and memory capabilities in ovariectomized rats. These effects may be associated with an anti-oxidative action of the extract.

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Key Words

neural regeneration; traditional Chinese medicine; Alzheimer's disease; Radix Achyranthis Bidentatae; ovariectomy; advanced glycation end products; cognition; learning and memory; oxidative stress; grants-supported paper; neuroregeneration

Research Highlights

(1) Kidney-tonifying recipe can reduce the accumulation of advanced glycation end products, prevent neuronal degeneration and improve cognitive functions in ovariectomized rats.
(2) Our experimental findings demonstrate that Radix Achyranthis Bidentatae can inhibit advanced glycation end product formation, reduce their levels in the frontal cortex, and improve learning and memory capabilities in ovariectomized rats.
(3) These novel findings provide insight into the mechanisms of action of Radix Achyranthis Bidentatae.

INTRODUCTION

The pathogenesis of Alzheimer's disease remains controversial, and increasing attention has been given to advanced glycation end products[1-9]. Glycation end products accumulate slowly in the normal body, and their levels increase with age. However, with age, the production of advanced glycation end products increases[10]. Advanced glycation end products can directly lead to the structural and functional impairment of nerve cells, induce oxidative stress through a receptor-mediated pathway, upregulate amyloid precursor protein expression, and promote inflammatory reactions, thereby inducing neuronal apoptosis[5-11]. Therefore, it is important to inhibit the formation of advanced glycation end products in Alzheimer's disease. The cognitive impairment in ovariectomized rats is associated with the accumulation of advanced glycation end products, neuronal degeneration in the hippocampus and cortex, as well as senile plaque formation[12-14]. Kidney-tonifying recipe can reduce the accumulation of advanced glycation end products, prevent neuronal degeneration and senile plaque formation, and significantly ameliorate the cognitive impairment in ovariectomized rats[12-14]. Radix Achyranthis Bidentatae, a single Chinese herbal constituent of the kidney-tonifying recipe, has a significant inhibitory effect on non-enzymatic glycation *in vitro* [12] and deserves further study.

This study aimed to determine whether Radix Achyranthis Bidentatae can inhibit formation of advanced glycation end products, reduce their contents in the hippocampus and frontal cortex, and improve learning and memory capabilities in ovariectomized rats. Furthermore, the mechanism underlying the *in vivo* inhibitory effect of Radix Achyranthis Bidentatae on advanced glycation end products was investigated.

RESULTS

Quantitative analysis of experimental animals

Thirty-two female Sprague-Dawley rats were equally and randomly divided into four groups: sham operation, ovariectomized, aminoguanidine and Radix Achyranthis Bidentatae groups. Excluding the sham operation group, rats in the remaining three groups were bilaterally ovariectomized. At 4 weeks after ovariectomy, the aminoguanidine group was given 0.1% aminoguanidine solution, the Radix Achyranthis Bidentatae group was given an alcohol extract of Radix Achyranthis Bidentatae containing 1.5 g/kg crude drug, and the ovariectomized and sham operation groups were given equal volumes of tap water for 12 weeks. All 32 rats were included in the final analysis.

Effect of Radix Achyranthis Bidentatae on advanced glycation end product content in the frontal cortex, hippocampus, urine and serum of ovariectomized rats

One-way analysis of variance showed that the levels of advanced glycation end products in the frontal cortex, urine and serum were significantly different among the various groups ($F_{3, 29}$ = 3.082, P = 0.043; $F_{3, 29}$ = 5.200, P = 0.005; $F_{3, 29}$ = 3.019, P = 0.048). No significant difference in content was found for the hippocampus ($F_{3, 29}$ = 2.919, P = 0.051). Differences in the average values between two groups were assessed with the least significant difference test. Compared with the sham operation group, levels of advanced glycation end products in the frontal cortex, hippocampus and urine were higher in the ovariectomized group (P = 0.203; P = 0.104; P = 0.730); serum level was significantly increased as well (P = 0.042). The advanced glycation end product content was significantly lower in the frontal cortex (P = 0.044; P = 0.025) and significantly higher in the urine (P = 0.026; P = 0.011) in the aminoguanidine group compared with the sham operation and ovariectomized groups. The aminoguanidine group showed a lower content of advanced glycation end products in the hippocampus compared with the ovariectomized group (P = 0.065), and a significantly higher content in the serum compared with the sham operation group (P = 0.042). There was no significant difference in serum levels between the aminoguanidine and ovariectomized groups (P = 0.153). The levels of advanced glycation end products in the frontal cortex were significantly lower in the Radix Achyranthis Bidentatae group compared with the sham operation and ovariectomized groups (P = 0.042; P = 0.024), while levels in serum were significantly higher compared with the sham operation group (P = 0.044). There was no significant difference in the serum levels of advanced glycation end products between the Radix Achyranthis Bidentatae and ovariectomized groups (P = 0.150; Figure 1).

Effect of Radix Achyranthis Bidentatae on markers of oxidative stress in ovariectomized rats

There were significant differences in the serum activities of superoxide dismutase and glutathione peroxidase, levels of malondialdehyde and activities of Mn-superoxide dismutase and CuZn-superoxide dismutase in the frontal cortex among the groups ($F_{3, 29}$ = 3.196, P = 0.038; $F_{3, 29}$ = 6.212, P = 0.002; $F_{3, 29}$= 5.562, P = 0.004;

$F_{3, 29}$ = 7.528, P = 0.001; $F_{3, 29}$ = 7.362, P = 0.001).

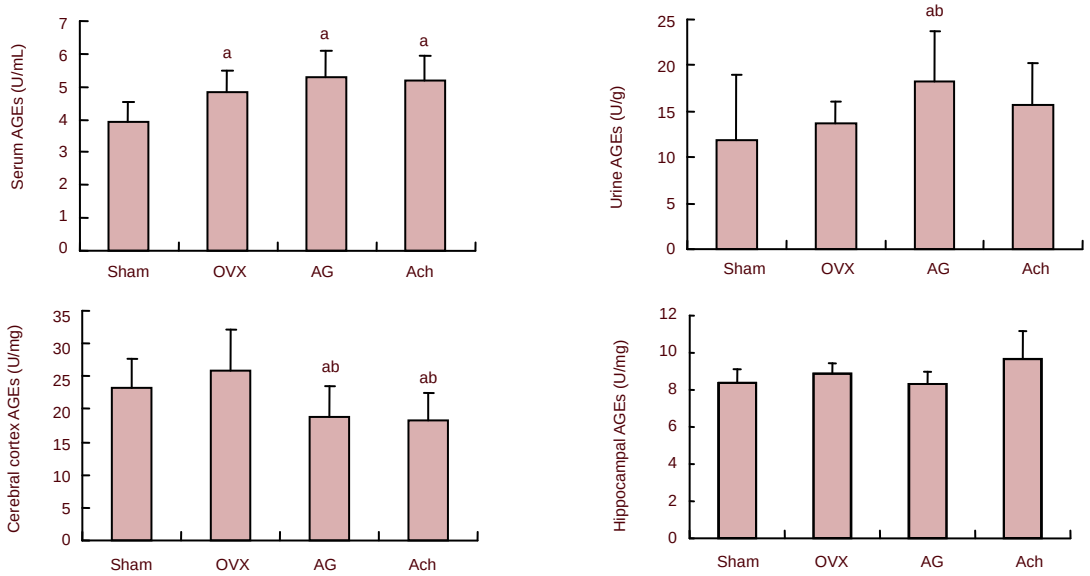

Figure 1 Effect of Radix Achyranthis Bidentatae (Ach) on advanced glycation end products (AGEs) in serum, urine, frontal cortex and hippocampus of ovariectomized rats.

Competitive enzyme-linked immunoabsorbent assay was used to determine levels of AGEs, and a standard curve was plotted using the logarithm of concentration of standard solution as the X axis and the absorbance value as the Y axis. The levels of AGEs in the sample were calculated with the linear regression method. Data are expressed as mean ± SD (n = 8). [a]P < 0.05, *vs*. sham operation group (Sham); [b]P < 0.05, *vs*. ovariectomized group (OVX). Differences between two groups were compared using the least significant difference test, and multiple comparisons were performed using one-way analysis of variance. AG: Aminoguanidine group.

Serum levels of malondialdehyde showed no significant differences among the groups ($F_{3, 29}$ = 1.119, P = 0.357), assessed with one-way analysis of variance. The comparison of the average between two groups showed that serum superoxide dismutase activity was significantly higher (P = 0.039), glutathione peroxidase activity was significantly lower (P = 0.048), malondialdehyde content in the frontal cortex was significantly lower (P = 0.017), and Mn-superoxide dismutase and CuZn-superoxide dismutase activities were significantly higher (P = 0.041; P = 0.018) in the ovariectomized group compared with the sham operation group. The aminoguanidine and Radix Achyranthis Bidentatae groups showed significantly lower serum superoxide dismutase activities (P = 0.026; P = 0.009), significantly higher glutathione peroxidase activities (P = 0.003; P = 0.010) and significantly lower Mn-superoxide dismutase activities in the frontal cortex (P = 0.000; P = 0.048) compared with the ovariectomized group. Furthermore, in the aminoguanidine group, malondialdehyde content in the frontal cortex was significantly higher (P = 0.005) and CuZn-superoxide dismutase activity was significantly lower compared with the sham operation group (P = 0.000). The Radix Achyranthis Bidentatae group showed no significant differences with the sham operation group in malondialdehyde levels or Mn-superoxide dismutase or CuZn-superoxide dismutase activities in the frontal cortex (P = 0.655; P = 0.121; P = 0.322; Figure 2).

Effect of Radix Achyranthis Bidentatae on learning and memory abilities in ovariectomized rats

One-way analysis of variance revealed significant differences in correct escape percentage and in escape latency among the groups ($F_{3, 29}$ = 3.196, P = 0.038; $F_{3, 29}$ = 4.180, P = 0.014). Compared with the sham operation group, correct escape percentage was markedly lower in the ovariectomized group (P = 0.040). In the aminoguanidine and Radix Achyranthis Bidentatae groups, correct escape percentage was significantly higher (P = 0.009; P = 0.044) and escape latency was significantly lower (P = 0.007, P = 0.018) compared with the ovariectomized group (Figure 3).

Correlation of advanced glycation end products with products of oxidative stress

Linear regression analysis showed that the levels of advanced glycation end products in the frontal cortex were significantly negatively correlated with the levels in serum and urine (r = –0.479, P = 0.010; r = –0.501, P = 0.005), and significantly positively correlated with

Mn-superoxide dismutase activity in the frontal cortex (r = 0.533, P = 0.002).

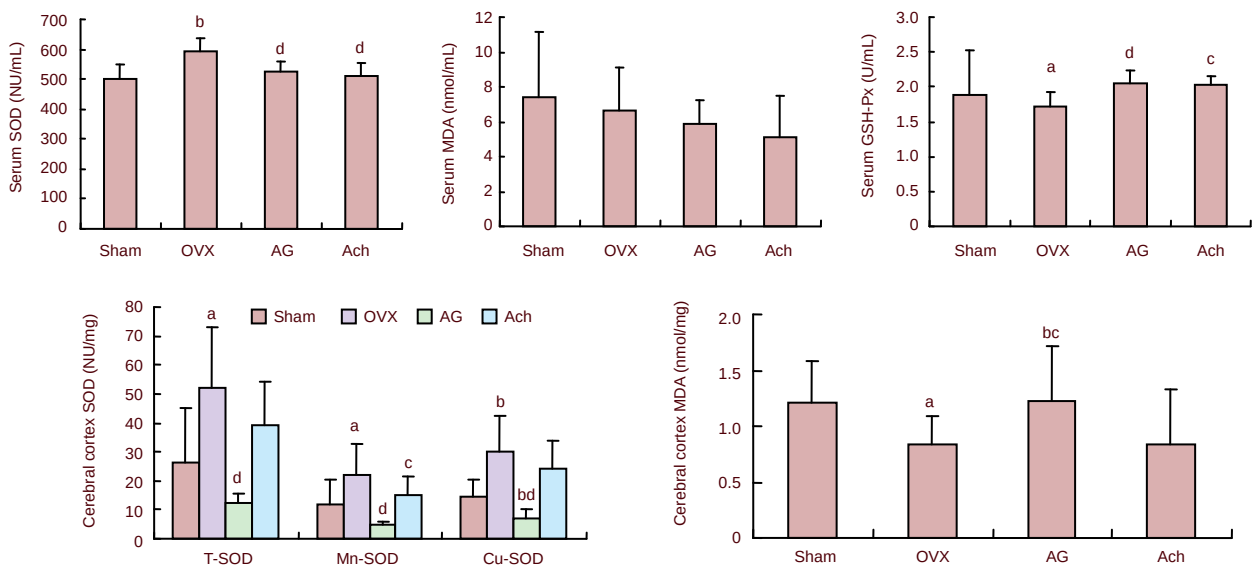

Figure 2 Effect of Radix Achyranthis Bidentatae (Ach) on serum superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), frontal cortex SOD and MDA levels in ovariectomized rats.

The levels of MDA were measured with the thiobarbituric acid colorimetric method. The SOD activity was measured with the improved pyrogallic acid autoxidation method. The GSH-Px activity was measured using 5,5′-dithiobis-(2-nitrobenzoic acid) with the direct method. Data are expressed as mean ± SD (n = 8). [a]P < 0.05, [b]P < 0.01, *vs*. sham operation group (Sham); [c]P < 0.05, [d]P < 0.01, *vs*. ovariectomized group (OVX). Differences between two groups were compared using least significant difference test, and multiple comparisons were performed using one-way analysis of variance. AG: Aminoguanidine group; T: total.

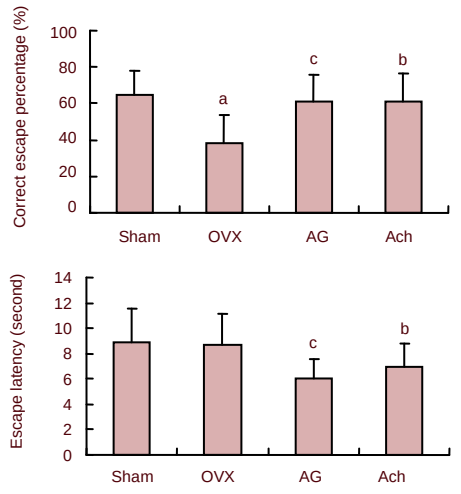

Figure 3 Effect of Radix Achyranthis Bidentatae (Ach) on correct escape percentage and escape latency in ovariectomized rats.

The correct behavior rate and escape latency of rats within 3 minutes were assessed with the Y-electrical maze. The higher correct behavior percentage and the shorter escape latency illustrate better learning and memory abilities. Data are expressed as mean ± SD (n = 8). [a]P < 0.05, *vs*. sham operation group (Sham); [b]P < 0.05, [c]P < 0.01, *vs*. ovariectomized group (OVX). Differences between two groups were compared using least significant difference test, and multiple comparisons were performed using one-way analysis of variance. AG: Aminoguanidine group.

However, they showed no significant correlation with malondialdehyde levels or CuZn-superoxide dismutase activity in the frontal cortex (r = 0.199, –0.219; P > 0.05; Table 1, Figure 4).

Table 1 Correlation analysis results (n = 32)

Index	Cerebral cortex AGEs	
	Pearson (r)	P
Serum AGEs	–0.479	0.010
Urine AGEs	–0.501	0.005
Cerebral cortex		
Mn-SOD	0.533	0.002
CuZn-SOD	0.199	0.245
MDA	–0.218	0.223

AGEs: Advanced glycation end products; SOD: superoxide dismutase; MDA: malondialdehyde.

DISCUSSION

The prevalence rate of Alzheimer's disease in females is 1.5–3.0 times higher than that in males. Estrogen levels decrease after menopause, greatly influencing cognitive functions and increasing the risk of Alzheimer's disease[1-4].

Therefore, estrogen deficiency in women has a major impact on the pathogenesis of Alzheimer's disease.

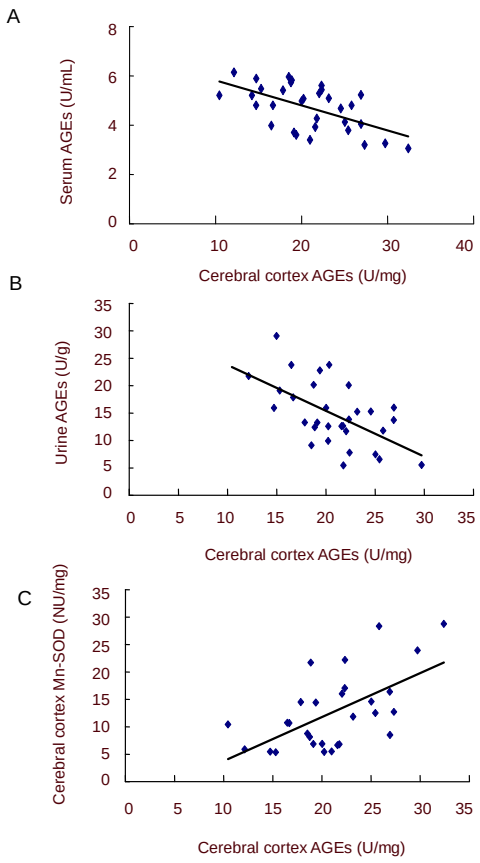

Figure 4 Correlation between advanced glycation end products (AGEs) in the frontal cortex, urine and serum, as well as Mn-superoxide dismutase (SOD) activity in the frontal cortex.

(A) Correlation of AGEs in the frontal cortex with those in serum (r = –0.479, P = 0.010).

(B) Correlation of AGEs in the frontal cortex with those in urine (r = –0.501, P = 0.005).

(C) Correlation of AGEs and Mn-SOD activity in the frontal cortex (r = 0.533, P = 0.002).

Ovariectomy and estrogen replacement can significantly impact the structure and function of the hippocampus and cortex, and lead to changes in cognitive function in animals[15-19]. Ovariectomy in rodents is considered a potential model of postmenopausal dementia[20-25]. Advanced glycation end products are the result of non-enzymatic protein glycation. In this process, free aldehydes or ketones in glucose react with protein amino groups through nucleophilic addition[26]. Advanced glycation end products are also carbonyl stress markers, and their formation results in protein modification, crosslinking of several glycation products and oxidative stress[27-28].

Therefore, these products are considered indicators of oxidative stress, aging and disease severity[29]. Advanced glycation end products play an important role in the pathogenesis of Alzheimer's disease, leading to structural and functional damage to nerve cells. These products induce oxidative stress *via* receptor pathways and promote amyloid precursor protein expression and the inflammatory response, ultimately causing neuronal apoptosis[5-11, 30-41].

There are several existing strategies for inhibiting the formation of advanced glycation end products. These include (1) inhibition of glycation: aminoguanidine, metformin, acarbose, rosiglitazone and other compounds[42]; (2) reducing the cross-linking of advanced glycation end products: the thiazole derivatives OPB-9195, ALT462 and ALT711[43]; (3) antioxidants: vitamin C, vitamin E, vitamin B1 and vitamin B6[44]; (4) blocking the interactions between advanced glycation end products and receptors[45]; and (5) Chinese herbal inhibitors[46-54]. Aminoguanidine and thiazole derivatives failed to pass stage III clinical trials because of adverse reactions and other factors. Chinese herbs have been used for more than 200 years in anti-aging treatment, and it is important to further investigate the effects of Chinese herbal inhibitors on advanced glycation end products to advance Alzheimer's disease therapy. Chinese herbs containing flavonoids, such as Quercetin, Rutin, Silymarin and Hesperidin, have shown great potential in inhibiting advanced glycation end product accumulation *in vitro* [46-50]. Flavonoids in Radix Puerariae, Mulberry Leaf, Radix Astragali and Gingko can diminish the accumulation of advanced glycation end products and inhibit lipid peroxidation. Ginseng, Radix Salvia Miltiorrhizae, Radix Bupleuri, Radix Rehmannia, Chinese Magnolivine Fruit, Asiatic Cornelian Cherry Fruit and hawthorn also show varying degrees of inhibition against non-enzymatic glycation *in vitro* [51]. In addition, Chinese herbal prescriptions inhibit advanced glycation end product accumulation and receptor expression in the myocardium, renal cortex and sciatic nerve of diabetic animals. These medicines include Composite *Tangkangming*, *Wenjintong*, *Xianzhen* Tablet, *Zhixiao Tongmaining*, sour herb compound and spleen and kidney-tonifying recipe[52-54].

The inhibitors of advanced glycation end products have been barely investigated. Radix Acanthopanacis Bidentatae contains carbohydrates, saponins, steroids and coumarin[55]. Among these, polysaccharides and ketosteroids are antioxidant components. Achyranthis Bidentatae polysaccharides can reduce oxidative stress in brain tissue of rats with cerebral trauma, and thus

protect the brain[56]. However, it is unclear whether it can inhibit non-enzymatic saccharification and prevent advanced glycation end product accumulation. Previous studies revealed that kidney-tonifying recipe reduces the production of advanced glycation end products, prevents neuronal degeneration and senile plaque formation, and significantly improves cognitive function in ovariectomized rats[13-14]. Radix Achyranthis Bidentatae, the active component of kidney-tonifying recipe, displays an inhibitory effect against non-enzymatic glycation *in vitro* [12]. Therefore, this study aimed to observe the effect of Radix Achyranthis Bidentatae on the learning and memory capabilities of ovariectomized rats.

Radix Achyranthis Bidentatae and aminoguanidine noticeably improved the performance of rats in the Y-maze test. The escape latency was shortened and the correct escape percentage was increased in rats given these medicines. In the normal human body, advanced glycation end products accumulate very slowly, except in some long-lived proteins, such as matrix proteins. When levels of carbohydrate or dicarbonyl compounds increase *in vivo*, advanced glycation end products also significantly increase[29]. These advanced glycation end products can accumulate in tissue macrophages and other scavenger cells through receptor and non-receptor pathways. The macromolecular advanced glycation end products are degraded into low-molecular-weight intermediates, and are then excreted by the kidneys[57]. Advanced glycation end products are composed of different components, and enzyme-linked immunosorbent assay was applied in this study to detect the Nε-(carboxymethyl)lysine epitope. Nε-(carboxymethyl)lysine is formed by an oxidative reaction during oxidative stress[58]. In our preliminary study[59], we found that serum levels of advanced glycation end products increased because of impaired glucose tolerance and oxidative stress after ovariectomy, and aminoguanidine treatment markedly decreased the advanced glycation end product content in the frontal cortex and hippocampus, while increasing levels in urine and serum. The levels in urine and serum were significantly negatively correlated with the levels in the frontal cortex. These findings demonstrate that the high levels of advanced glycation end products in the frontal cortex make their way into the blood. Radix Achyranthis Bidentatae treatment markedly decreased the concentration of advanced glycation end products in the frontal cortex, while elevating levels in urine and serum, with no significant difference from the aminoguanidine group. Radix Achyranthis Bidentatae alcohol extracts dose-dependently inhibit non-enzymatic glycation *in vitro* [12], with 54.23% inhibition at a dose of 0.1 mg/mL (with aminoguanidine as positive control). This is evidence that both aminoguanidine and Radix Achyranthis Bidentatae inhibit the generation of advanced glycation end products, facilitate their degradation, increase their transfer into blood and decrease their levels in the brain, thereby protecting neurons[60].

Estrogen is a phenolic compound that has antioxidant effects through the mitogen-activated protein kinase and nuclear factor kappa B pathway. It also activates the estrogen receptor and promotes superoxide dismutase synthesis[61-63]. Superoxide dismutase is a major antioxidant; it can catabolize the toxic superoxide radical (O_2^-) into hydrogen peroxide and oxygen. Superoxide dismutase exists as three isozymes, in which Mn-superoxide dismutase is associated with mitochondrial oxidative damage[61-63]. At 16 weeks after ovariectomy, the levels of advanced glycation end products in the frontal cortex and hippocampus began to rise, although not significantly, and CuZn-superoxide dismutase and Mn-superoxide dismutase activities increased significantly. Concomitantly, levels of malondialdehyde decreased. Mn-superoxide dismutase activity was significantly positively correlated with advanced glycation end product levels in the frontal cortex. These findings suggest that reduced levels of the antioxidant estrogen result in increased protein carbonyl levels, increased concentrations of advanced glycation end products, increased activity of mitochondrial and cytoplasmic antioxidant enzymes, neuronal degeneration, and learning and memory dysfunction. In addition, we also found that while levels of oxidative stress increased, levels of malondialdehyde in the frontal cortex decreased, suggesting that, although saccharification and protein oxidation increased, there was a decrease in lipid peroxidation. Zhang *et al* [64] showed that early memory impairment prior to senile plaque formation is negatively correlated with protein carbonyl complex and advanced glycation end product levels in APPswe/PS1dE9 mice. We obtained similar results, except that Mn-superoxide dismutase levels were positively associated with memory impairment. The correlation between oxidative stress levels and superoxide dismutase content remains elusive. Eynan *et al* [65] proposed that increased Mn-superoxide dismutase activity is possibly a stress response of mitochondria in response to oxygen free radical damage, and therefore can indicate high oxidative stress levels. We speculate that, although there is a rise in Mn-superoxide dismutase activity, the increase in enzyme activity is much lower

than the increase in the rate of O_2^- production. Consequently, the susceptibility of mitochondria to damage increases.

In this study, advanced glycation end product levels in the frontal cortex and hippocampus showed no significant changes after ovariectomy, oxidative stress levels significantly increased, and Mn-superoxide dismutase activity was significantly positively correlated with advanced glycation end product concentration in the frontal cortex, and neuronal degeneration and cognitive impairment were observed. Our experimental findings are consistent with the results of Srikanth *et al*[66]. In the early stage of Alzheimer's disease, although the advanced glycation end product level is very low, it may disrupt different receptor signaling pathways, ultimately leading to neuronal dysfunction and neurodegeneration. Advanced glycation end products are the result of oxidative and carbonyl stress, and can accelerate neuronal degeneration and cause Alzheimer's disease. Our study showed that Radix Achyranthis Bidentatae treatment significantly decreases serum superoxide dismutase activity, significantly increases glutathione peroxidase activity, and significantly reduces Mn-superoxide dismutase activity in the frontal cortex of ovariectomized rats. The Radix Achyranthis Bidentatae inhibitory effect on advanced glycation end product accumulation is associated with an antioxidative effect. Radix Achyranthis Bidentatae can protect brain tissue against oxidative stress injury caused by free radicals and advanced glycation end products.

The polysaccharides and ketosteroids in Radix Achyranthis are antioxidants[55-56]; however, they require further research to clarify their roles in the antioxidative effects of Radix Achyranthis. Therefore, further investigation of the natural active constituents of Radix Achyranthis Bidentatae is strongly warranted for the prevention and treatment of Alzheimer's disease.

In summary, Radix Achyranthis Bidentatae can improve learning and memory abilities, reduce levels of advanced glycation end products, and protect brain neurons in ovariectomized rats. The effective components of Radix Achyranthis Bidentatae responsible for the antioxidative effects of the herbal medicine are promising candidate drugs in the development of non-enzymatic glycation inhibitors.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

The experiment was performed from May 2011 to May 2012 in the Experimental Animal Center and Pathology Laboratory of Beijing University of Chinese Medicine, China.

Materials

Animals

Thirty-two healthy female Sprague-Dawley rats, aged 9 months, weighing 320–380 g, and of specific pathogen free grade, were provided by Vital River Laboratory Animal Technology Co., Ltd., Beijing, China (license No. SCXK (Beijing) 2007-0001). Animals were housed and fed in cages in a room at 24°C under a 12-hour light/dark cycle. All procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[67].

Drugs

Radix Achyranthis Bidentatae was provided by Beijing Tongrentang Company, China and produced in Henan Province, China. It is the dried root of *Chyrabthes Bidentata* BL., identified by Professor Chunsheng Liu from Beijing University of Chinese Medicine School of Pharmacy. Radix Achyranthis Bidentatae decoction pieces were extracted three times using 10 mL 75% ethanol per gram, 2 hours each, and then centrifuged at 4 000 × *g* for 10 minutes. The centrifugal supernatant was collected, alcohol was recovered until there was no alcohol smell, and the concentration of the crude drug was calculated. The drug containing Radix Achyranthis Bidentatae at 1 g/mL was stored at –4°C in a refrigerator. Prior to use, the extract was resuspended to the required concentration.

Methods

Preparation of ovariectomized model and drug administration

In the ovariectomized, aminoguanidine and Radix Achyranthis Bidentatae groups, a dorsal incision was opened under anesthesia, and rats were bilaterally ovariectomized. In the sham operation group, an equal volume of adipose tissue was dissected out. At 4 weeks after ovariectomy, rats in the aminoguanidine group were given 0.1% aminoguanidine (Sigma, St. Louis, MO, USA) aqueous solution, while the Radix Achyranthis Bidentatae group was intragastrically administered the alcohol extract of Radix Achyranthis Bidentatae (containing 1.5 g/kg crude drug, equivalent to a clinical single prescription[68]). The ovariectomized and sham operation groups were

intragastrically administered the same volume of tap water. The treatment was given for 12 weeks.

Assessment of learning and memory capabilities using the Y-electrical maze test

The Y-electrical maze was provided by Shanghai Bio-will Co., Ltd., Shanghai, China, and consisted of a trisection radial labyrinth box and control instrument[69]. The bottom of the box was covered with an electric grid with a wire diameter of 0.2 cm, length of 14 cm, and an interval of 1 cm between wires. Each arm was 45 cm long, with a 15-W stimulus signal light on the top. The control instrument included a voltage control button, a delay control button, an automatic control button and I, II, III and 0 buttons. When the I, II or III buttons were pressed, the signal lights in the corresponding arm became lit, and the arm was switched off and became a safe zone (red light area), while the other two arms without lights and the junction area were energized and were unsafe areas (electric shock area). When the 0 button was pressed, none of the three arms were electrified, but the junction area was electrified. The experiment was performed within 2 days using the random restless method. The rats were trained on day 1 and maintained on day 2. Rats were placed into the maze to adapt for 3–5 minutes prior to the experimentation. The safe zone was alternated randomly to train for the ability to distinguish the light stimuli and find the safe position. After the electric shocks, the rats escaped towards the safety zone, with light on for 10–15 seconds, and the test was over when the light was turned off. The arm where the rat was situated was the starting point of the next test, and the rat was allowed to rest for 30 seconds or 1 minute before the next test. Rats were trained 20 times on day 1 and tested for 24 hours on day 2 to maintain memory ability. The percentage of correct behavior within 3 minutes (number of correct responses over the total number of responses) and escape latency (time required for the rats to escape to the area where the lights were turned on) were determined.

Sample preparation

Rats were placed in metabolic cages to collect 24-hour urine, and then the femoral artery bleed was collected, and the serum was separated, packaged, and preserved at –20°C until use. The rat brains were rapidly stripped at 4°C, and the frontal cortex and hippocampal tissues were stored at –80°C.

Advanced glycation end product content as detected by competitive enzyme-linked immunoadsorbent assay

Hippocampus and frontal cortex tissue homogenates were prepared, and competitive enzyme-linked immunoadsorbent assay was performed as previously described[70]. Hippocampus and cerebral cortex tissue (30 mg) were rinsed with 300 mL 0.2 mmol/L PBS containing 2% sodium dodecylsulfate and 0.1 mol/L β-mercaptoethanol (Sigma), boiled for 10 minutes, centrifuged at 10 000 × *g* for 10 minutes, and resuspended three times with 0.1 mol/L PBS. Subsequently, tissues were dissolved in 30 μL 0.1 mol/L PBS containing 0.1% proteinase K and digested at 37°C overnight. After the supernatant was discarded, samples were diluted 1:1 000, and advanced glycation end product content was determined. Serum was diluted 1:400, and urine was diluted 1:10 after centrifugation. Polyclonal antibody against advanced glycation end products was prepared as previously described[71]. The dilutions of advanced glycation end products including human serum albumin (self-made), rabbit anti-advanced glycation end products-bovine serum albumin (self-made) and enzyme-labeled goat anti-rabbit IgG (Boster, Wuhan, Hubei Province, China) were determined with the chessboard titration method[71]. For the detection, advanced glycation end products-human serum albumin was diluted with carbonate buffer (pH 9.5) to 5 μg/mL and was used to coat a 96-well reaction plate, 100 μL/ well, at 4°C overnight. The plate was then rinsed with Tween 20 and PBS six times, 5 minutes each, and incubated with blocking solution 200 μL/well (containing 0.5% gelatin and 0.02% sodium azide, PBS pH 7.4) at 37°C for 2 hours. Then, advanced glycation end product human serum albumin (standard curve), at a concentration of 0.05–400 μg/mL, was mixed with the experimental samples and an equal volume of advanced glycation end product bovine serum albumin antibody (1:2 000) and incubated for 1 hour at 100 μL/well. Samples were then incubated with 100 μL/well horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2 500) at 37°C for 1 hour, and then with 100 μL/well ortho-phenylenediamine at 37°C for 20 minutes. The reaction was terminated with 50 μL/well 2 mol/L H_2SO_4, and the absorbance value at 492 nm was read using an automatic enzyme immunoassay instrument (Multiskan Ascent, Helsinki, Finland). The standard curve was plotted with the logarithm of the concentration of the standard solution as the X axis, and the absorbance value as the Y axis. The advanced glycation end product concentration was calculated with the linear regression method. 1 U advanced glycation end product is equivalent to the absorbance value of 1 μg/mL advanced glycation end product-bovine serum albumin.

Measurement of superoxide dismutase,

malondialdehyde and glutathione peroxidase

Serum superoxide dismutase activity, malondialdehyde content and glutathione peroxidase activity were measured with chemical colorimetric methods. CuZn-superoxide dismutase and total superoxide dismutase activities (Mn-superoxide dismutase activity = total super superoxide dismutase activity – CuZn-superoxide dismutase activity), as well as malondialdehyde level in the frontal cortex homogenate supernatant were also assayed. The superoxide dismutase activity was detected with the improved pyrogallic acid autoxidation method; when the superoxide dismutase inhibition rate reaches 50% per mL of reaction liquid, superoxide dismutase activity is determined and represented as a nitrite unit[72]. The levels of malondialdehyde were measured with the thiobarbituric acid colorimetric method[72]. Glutathione peroxidase activity was measured using 5,5′-dithiobis-(2-nitrobenzoic acid) with a direct method[72]. All assay kits were provided by Nanjing Jiancheng Biological Institute, Nanjing, Jiangsu Province, China.

Statistical analysis

Data are expressed as mean ± SD, and statistically analyzed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Differences among groups were compared using one-way analysis of variance, and average values between two groups were compared with the least significant difference test. Correlations between advanced glycation end products, superoxide dismutase and malondialdehyde were analyzed using linear regression analysis. A $P < 0.05$ value was considered to indicate significant difference.

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