

Effects of timosaponins on learning and memory abilities of rats with dementia induced by lateral cerebral ventricular injection of amyloid β -peptide

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Abstract: **Objective** To investigate the effects of timosaponins, one group of the two major components of *Anemarrhena asphodeloides* Bge, on the learning and memory capacities of rats with dementia induced by amyloid β -peptide (25-35) [$A\beta$ (25-35)]. **Methods** Sixty SD rats were randomized into 6 groups ($n=10$) and except for those in the control group, all other rats were subjected to lateral cerebral ventricular injection of aggregated $A\beta$ (25-35) to prepare rat models of dementia. Twenty-four hours after the injection, the rats received intragastric administration of timosaponins at 3 different doses (treatment group) or *Ginkgo biloba* extract EGB761 on a daily basis for 14 consecutive days. From postoperative days 8 to 14 after $A\beta$ (25-35) injection, Morris water maze test was performed to evaluate the effects of $A\beta$ (25-35) and the therapeutic agents timosaponins on the learning and memory capacity of the rats. On day 14, the level of malonaldehyde (MDA), superoxide dismutase (SOD) activity and total antioxidation capacity in the brain tissue of the rats were measured. **Results** $A\beta$ (25-35) induced significant learning and memory impairment in the rats, which had lowered SOD activity and total antioxidation capacity ($P<0.01$) with elevated MDA level ($P<0.05$). Compared with the rats in dementia model group, those receiving timosaponin treatment at different doses all manifested alleviation of learning and memory impairment ($P<0.05$), with enhanced SOD activity ($P<0.05$) and total antioxidation capacity ($P<0.01$) and reduced MDA level ($P<0.05$) in the brain tissue. **Conclusion** Timosaponins can remarkably enhance the learning and memory capacities in rats with $A\beta$ (25-35)-induced dementia, presumably in relation to their actions to promote the scavenging of the free radicals.

Key words: timosaponins; amyloid β -peptide; Morris water maze; dementia; free radicals

Extracellular senile plaques composed of amyloid β -peptide ($A\beta$), intracellular neurofibrillary tangles, and extensive drastic neuronal and synaptic degeneration constitute the major pathological hallmarks of Alzheimer's disease (AD). Increasing experimental as well as genetic evidence supports a causal role of $A\beta$ in the pathogenesis of AD, and the senile plaques containing $A\beta$ as the core protein are often closely associated with neuronal degeneration^[1]. A number of studies have shown that high concentrations of $A\beta$ are toxic and may cause damage of the biomacromolecules. $A\beta$ is capable of inducing neuronal apoptosis^[2] and inhibiting cellular redox activity^[3], which are believed to be dependent on the peptide assembly state such as its solution or aggregation^[4]. With recent findings suggesting links between AD, deposition of $A\beta$ and oxidative stress^[5], much attention is given currently to antioxidant

research. The free-radical theory of aging^[6] suggests that cumulative oxidative damage at the cellular and tissue level arises as a consequence of normal aerobic metabolism^[7].

In traditional oriental medicine, quite a few herbal drugs have been used for centuries for treating disorders associated with aging, causing generally less side effects and consequently ensuring safe application. *Ginkgo biloba*, whose existence can be date back to the time of the dinosaurs, is one of the oldest tree still thriving on the planet^[8]. In European countries, the extract of *Ginkgo biloba* is prescribed for the treatment of peripheral and cerebral blood insufficiency. Experimental evidence suggests that this extract inhibits blood coagulation^[9], attenuates age-related deterioration of cognitive functions in rats^[10], and improves degenerative dementias of the Alzheimer's and multi-infarct types^[11,12]. Existing pharmacological data and clinical trials have demonstrated *Ginkgo biloba* extract EGb 761 to be a potent antioxidant agent and free radical scavenger with neuroprotective effects^[13-16]. *Anemarrhena asphodeloides* Bge, a traditional Chinese herbal drug, is derived from the dry rhizoma of the plant *Anemarrhena asphodeloides*, a species of *Liliaceae*. Anemarrhena saponins

Received:2004-11-15

Supported by National 863 Program of China for New Drug Research and Development (2003AA2Z3535) and Natural Science Foundation of Guangdong Province (2KB04207S)

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and timosaponins are the main active components of this drug. Sapogenins from *Anemarrhean asphodeloides* was reported to improve the learning and memory abilities with also effect to increase the levels of brain-derived neurotrophic factor (BDNF) in aged rats^[17]. In addition, our previous study showed that timosaponins could increase cellular level of nicotinic acetylcholine receptors in rat brains in a dose-dependent manner^[18]. Timosaponin also inhibits N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced generation of superoxide in leukocytes^[19] *in vitro* and lower blood glucose concentration^[20,21]. Recent study suggests that timosaponin is capable of some estrogen-like actions^[22] to serve as an important protective agent against AD^[23].

The A β peptide fragment A β (25-35) has been shown to be directly toxic to neurons and able to increase the vulnerability of neurons to other insults^[24]. According to Varadarajan *et al*^[25], this truncated peptide is more rapidly toxic and causes more oxidative damage than A β (1-42). Additionally, Giovannelli *et al* reported the onset of impairment of object recognition in the first two weeks after A β (25-35) injection at the nucleus basalis in rats, whereas A β (1-40) impaired the performance till two months after the injection^[26]. This study was designed to determine whether timosaponins (timosaponin B- II, timosaponin E1 and timosaponin B) may antagonize the toxicity of A β (25-35) in a rat model of learning and memory dysfunction induced by microinjection of A β (25-35) into the lateral cerebral ventricles.

MATERIALS AND METHODS

Rats

Totally 60 adult male Sprague-Dawley rats with body weight ranging from 200 to 250 g were purchased from the Experimental Animal Center, Southern Medical University (certificate No. 2003A073). The rats were kept in cages (5 in each cage) with the temperature controlled at 24 \pm 2 °C, relative humidity of (60 \pm 10)%, and in 12-hour light-dark cycles. Free access to food in the form of dry pellets and water was allowed. The animal experiments were performed according to the internationally accepted ethical guidelines.

Drugs

The timosaponins were extracted from *Anemarrhena asphodeloides* Bunge (purchased from Hebei Province, China) in our laboratory. The

timosaponins, with the main components of timosaponin B- II, timosaponin E1 and timosaponin B, were dissolved in sterile distilled water at different concentrations. Ginkgo biloba extract EGB761 serving as a positive therapeutic control drug was purchased from Cerenin, Dr. Willmar Schwabe Co., (Germany), also suspended with sterile distilled water. A β (25-35) (Sigma Chemical Co, USA) was dissolved in sterile distilled water, and stored at -20 °C. To obtain the neurotoxic form of A β (25-35), the peptide solution was incubated at 37 °C for 7 days^[26]. Superoxide dismutase (SOD), malondialdehyde (MDA), total antioxidation capacity and protein content test kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Induction of learning and memory dysfunction

The rats were anesthetized with 10% chloral hydrate (0.35g/kg) and fixed in a stereotaxic apparatus for microinjection of A β (25-35) into the lateral cerebral ventricle. The bregma and the skull surface served as the stereotaxic zero points^[27,28]. The dura mater was surgically exposed, and the needle was lowered into the right lateral ventricle [posterior (P) 1.3mm, lateral (L) 1.3 mm, ventral (V) 4.0 mm]^[29], where 1 μ l A β (25-35) (10 mmol/L) or sterile distilled water was slowly infused within 5 min, and the syringe was not withdrawn until 5 min after the injection. The therapeutic drug administration was performed 24 h after the surgery, once a day for 14 consecutive days.

Animal grouping

The rats were randomly divided into 6 groups to receive intracerebral ventricular injection (i.c.v.) and intragastric administration (i.g.) of distilled water (control group), i.c.v. of A β (25-35) with i.g. of distilled water (dementia model group), i.c.v. of A β (25-35) and i.g. of EGB761 (EGB761 group), or i.c.v. of A β (25-35) and i.g. of three doses (50,100 and 200 mg/kg, designed according to the results of our previous study) of timosaponins (treatment groups).

Morris water maze test

The rats were trained in Morris water maze test for 7 consecutive days from days 8 to 14 following A β (25-35) injection. The water maze apparatus consisted of a circular pool filled with water 30 cm in depth, and a circular platform supported by a base resting on the bottom of the pool, 2 cm below the surface of the water. The water was mixed with milk for the coloring and the

rat heads were stained black. The platform resided in the center of the northwest quadrant. At the start of the test, the rats were made to face the wall, and when they managed to locate the platform, they were allowed to remain on it for 15 s. If the rats failed to locate the platform within 2 min, they were removed from the water and placed on the platform for 15 s. Data collection was automated by an on-line video tracking device designed to track the object with the highest contrast, which rendered only the black heads of the rats visible against the milk-white background. The escape latency (time to locate the platform) and swimming path were recorded.

Assay of SOD, total antioxidation capacity and MDA

Two weeks after distilled water and A β (25-35) injection and corresponding treatment, the rats were killed by decapitation and the brain tissue was taken to prepare 10% tissue homogenate by addition of 0.86% cold saline (pH 7.4) and stored at -30°C for later examination. Total antioxidation capacity and MDA assays were performed according to the instructions of the test kits and the tissue homogenate was diluted to 2% for SOD test. MDA was quantified by thiobarbituric (TBA) method to measure the absorbance at 532 nm (expressed as nmol/mg.protein). In SOD assay, xanthine oxidase was used as a superoxide generator to reduce nitroblue tetrazolium (NBT), which was inhibited by SOD present in the solution. This inhibition of NBT reduction into blue-colored formozan in the presence of phenazine metha sulphate (PMS) and NADH was measured at 560 nm. One unit of enzyme activity was defined as the amount of enzyme that inhibited the rate of reaction by 50% in 1 min under the defined assay conditions and the result expressed as units (U) of SOD activity/mg.protein. The antioxidant defense system consisted of enzymatic and non-enzymatic antioxidants, which were able to reduce Fe^{3+} to Fe^{2+} . The total antioxidation capacity was measured by the reaction of phenanthroline and Fe^{2+} using a spectrophotometer at 520 nm. At 37°C , a total antioxidation capacity unit was defined as the amount of antioxidant required to produce an absorbance increase of 0.01 in 1 mg protein.

Statistical analyses

The data were presented as $Mean \pm SD$. The data between the model group and the control group were analyzed with independent-sample t test, and those

between the model group, the treatment groups and EGB761 group were analyzed with one-way ANOVA (least-significant difference test) using SPSS 10.0 software. A P value less than 0.05 was considered to indicate statistically significant difference.

RESULTS

Effect of timosaponins on learning and memory dysfunction (Morris water maze test)

At the beginning of the training, most of the swimming tracks of the rats presented a random pattern near the edge of the pool, instead of a shorter linear path. Towards the end of the 7-day training, most of the rats, with the exception of the rats in the model group, managed to find the platform through a roughly linear path. Typical tracks of the rats in the control, model and treatment groups in the Morris water maze are shown in Fig.1. Apparently, the mean latencies were greatly longer in the model group than in the control group, or in other words, A β (25-35)-treated rats took longer time to find the platform than the control rats did. Treatment with timosaponins and Egb761 for 14 days could significantly shorten the latencies, which showed no significant difference between Egb761 and treatment groups (Tab.1).

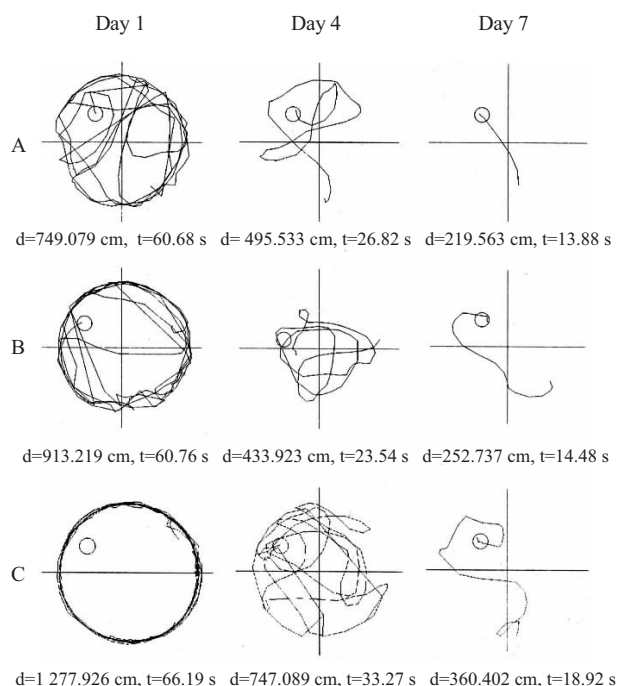


Fig.1 Typical examples of tracks made by the rats in the 4 groups in the Morris water maze test

A: Control group; B: Treatment group (100 mg/kg); C: Dementia model group. t: Latent period; d: Distance traveled

Tab.1 Latencies of the rats to reach the platform in Morris water maze test

Group	n	Latency (Mean±SD)
Control	8	26.01±4.79**
Model	8	33.59±8.29
Egb761 (15.5 mg/kg)	8	26.04±5.55 [#]
Timosaponins (mg/kg)		
50	9	24.34±5.13**
100	8	25.70±4.11 [#]
200	8	24.84±5.45**

[#]*P*<0.05, ***P*<0.01 vs model group. The data between the model and the control groups were analyzed with independent-sample *t* test, and those between the model, treatment, and EGB761 groups with one-way ANOVA (LSD) by SPSS 10.0 software.

Tab.2 SOD activity, MDA level and total antioxidation capacity in the brain tissue of rats (Mean±SD)

Group	n	SOD (U/mg.pro)	MDA (nmol/mg.pro)	Total antioxidation capacity (U/mg.pro)
Control	9	302.41±43.46**	4.60±1.09 [#]	8.37±1.67**
Model	10	229.75±34.13	5.95±1.37	2.75±1.18
Egb761 (15.5 mg/kg)	9	298.51±45.40**	4.80±1.11 [#]	4.50±2.30 [#]
Timosaponins (mg/kg)				
50		275.53±31.90 [#]	4.94±0.69 [#]	4.19±1.04
100	10	277.23±63.55 [#]	4.67±0.95**	8.39±2.37**
200	10	276.25±32.57 [#]	4.85±0.77 [#]	9.48±1.94**

[#]*P*<0.05, ***P*<0.01 vs model group. Statistical analysis of the data is specified in Tab.1.

SOD activity, total antioxidation capacity and MDA level in brain tissue

After treatment with Aβ (25-35), the activity of SOD and total antioxidation capacity in the rat brain tissues were lowered and MDA level increased compared with those in the control rats. Compared with the model group, the rats receiving timosaponins and Egb761 therapies showed remarkably increased SOD activity and total antioxidation capacity as well as reduced MDA level in the brain tissue (Tab.2).

DISCUSSION

The results of this study demonstrated that a single acute injection of Aβ (25-35) into the lateral cerebral ventricle induced marked amnesic effect in rats as shown by the repeated training of the rats for spatial recognition in Morris water maze, one of the most widely employed paradigms to assess learning and memory function in rodents [30]. Aβ (25-35), as we observed, induced impairment of the learning and memory abilities of the rats, and the rats in model group tended to circle around the pool whereas the control rats swam more directly to reach the platform in the later training days. Excessive oxygen-derived free radicals have been recognized for their potential to damage the neurons and reduce the density of neurons in the brain, leading to deteriorated learning and memory capacity in animals [31]. The central nervous system is especially vulnerable to free radical damage due to the high demand of oxygen consumption of the brain, its abundant lipid content, and the relative paucity of antioxidant enzymes compared with other tissues [32]. Aβ in the brain of AD patient may promote the production of free radicals, which at least partially mediated the cytotoxic effects of Aβ on the neurons [33].

Some studies have also found that Aβ is directly responsible for free-radical damage to the neuronal membrane systems, leading to subsequent neuronal loss in the AD brain [34]. Free radical attack on phospholipid polyunsaturated fatty acids (PUFA) can ultimately lead to the production of multiple aldehydes with different carbon chain length, including MDA [35]. In a sense, the level of MDA can be taken as an indicator for the state of lipid peroxidation. SOD converts superoxide radicals to H₂O₂, which is in turn broken down to water and oxygen in the presence of catalases. Thus, SOD and the catalases constitute the first defense mechanism against reactive oxygen species. A decrease in the activity of the antioxidant system would most likely lead to cellular death. Marcus *et al* [36] observed significantly decreased SOD activity in frontal and AD temporal cortex. Our present study showed that in rats treated with Aβ (25-35), the activity of SOD and the total antioxidative capacity decreased and the level of MDA increased, suggesting that Aβ (25-35) could induce the overproduction of free radicals and this free radical metabolism disorder caused impairment of the brain function.

As the extract of a herbal drug with proven

efficacy and safety, timosaponins, according to the results of this present study, resulted in a significant improvement of learning and memory capacity of rats treated with A β (25-35) with such potency as comparable to that of EGB761. At the same time, timosaponins significantly enhanced SOD activities and total antioxidation capacity and decreased MDA level, similar to the effect of EGB761. These results demonstrate that timosaponins can remarkably enhance the learning and memory capacities, presumably through promoting scavenging of the free radicals. We had presumed that timosaponins possessed such potential to act on multiple targets, and our previous study also showed that timosaponins could ameliorate the dysfunction of the cholinergic system^[18]. Timosaponins may still have some other potentials as intervening in cell apoptosis, a probability that is currently under investigation.

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知母皂苷元对痴呆模型大鼠的影响

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摘要:目的 建立大鼠 β -淀粉样蛋白[A β (25-35)]诱导的痴呆模型, 探讨知母总皂苷在阿耳茨海默病(AD)发病机制中的作用。方法 SD 大鼠 60 只, 采用脑立体定位技术在大鼠单侧侧脑室一次性注射老化的 A β (25-35)建立实验性 AD 模型。手术 24 h 后, 大鼠经灌胃分别给予 3 种剂量的知母总皂苷和 EGB761, 模型组和空白组给予等剂量的蒸馏水, 1 次 / 1 d, 连续 14 d。在给予 A β (25-35)后的第 8 天, 应用 Morris 水迷宫法测定大鼠的学习记忆功能, 造模后第 14 天断头取脑, 测定脑组织超氧化物歧化酶(SOD)、丙二醛(MDA)和总抗氧化能力。结果 与空白对照组相比, 模型组大鼠空间学习记忆能力明显减弱, 寻找平台潜伏期明显延长 ($P < 0.01$); 脑组织 SOD、总抗氧化能力明显降低 ($P < 0.01$), 而 MDA 明显升高 ($P < 0.05$)。与模型组比较, 给予知母总皂苷治疗后的大鼠学习记忆功能障碍得到明显改善, 潜伏期显著缩短 ($P < 0.05$), SOD 活力显著提高 ($P < 0.05$), 总抗氧化能力也显著增强 ($P < 0.05$), MDA 水平明显降低 ($P < 0.05$)。结论 知母总皂苷可明显改善 AD 模型大鼠的学习记忆功能, 同时可提高模型大鼠的抗氧化能力, 这有可能是其改善学习记忆的原因之一。

关键词: 知母总皂苷; β 样淀粉蛋白; Morris 水迷宫测试; 痴呆; 自由基

中图分类号: R284.1; R392.11; R749.16 文献标识码: A 文章编号: 1000-2588(2005)02-0121-06