Neurosci Bull December 1, 2009, 25(6): 391-396. http://www.neurosci.cn
DOI: 10.1007/s12264-009-0601-4

·Original Article·

Tetrahydroxy stilbene glucoside reduces the cognitive impairment and overexpression of amyloid precursor protein induced by aluminum exposure

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Abstract: Objective Excessive aluminum (Al) exposure impairs neurocognitive function in humans and animals. Epidemiologic studies have shown a potential linkage between chronic Al exposure and Alzheimer's disease. The present study aims to evaluate the effects of tetrahydroxy stilbene glucoside (TSG), the extract from herbal medicine *Polygoni Multiflori*, on cognitive impairment and the over-expression of hippocampal amyloid precursor protein (APP) induced by chronic exposure to Al in rats. **Methods** Rats were treated with 0.3% aluminum chloride ($AlCl_3$) prepared in the drinking water for 90 d. $AlCl_3$-treated animals were then randomly assigned to receive vehicle, TSG (4 g/kg), or Vitamin E (VE; 40 mg/kg) treatment for 5 months. VE served as a positive control. The effect of TSG was evaluated by passive avoidance task, and APP expression was evaluated by Western blotting. **Results** Following exposure to $AlCl_3$ for 90 d, animals displayed a striking decrease (>80%) in step-through latency in the passive avoidance task and a significant increase in the expression of APP in the hippocampus. Both TSG and VE significantly ameliorated the performance impairment in the passive avoidance task, and suppressed the over-expression of APP. Moreover, the effects of TSG, but not of VE, were in a time-dependent manner. **Conclusion** TSG may possess therapeutic effects against Alzheimer's disease.

Keywords: tetrahydroxy stilbene glucoside; cognitive impairment; amyloid precursor protein; aluminum

1 Introduction

Alzheimer's disease (AD) is an epidemic increasingly threatening the public health, while its cause has not yet been fully understood[1]. In the past 3 decades, accumulating evidence has suggested that exposure to a high level of aluminium (Al) leads to neurofibrillary degeneration, and that Al concentration is increased in degenerating neurons in AD [2]. The role of Al in the etiology and pathogenesis of AD has drawn more attention, due to the well documented Al-induced cognitive impairment from clinical observations[3] and animal experiments[4]. The amyloid cascade hypothesis proposes that accumulation of Aβ derived from proteolytic cleavage of amyloid precursor protein (APP) in the brain is the primary stimulator for AD pathogenesis[5]. Studies have found that Al could alter the conformation of Aβ and enhance its aggregation on the surface of cultured neurons[6], the process of which may precede the formation of intraneuronal neurofibrillary tangles and neuritic plaques[7], both of which are the neuropathological features of AD. Based on these findings, the potential linkage between Al and AD is reconsidered[8]. Although the mechanisms of Al neurotoxicity are not fully understood, multiple pathways are likely to be involved in

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Article ID: 1673-7067(2009)06-0391-06
CLC number: R749.1+6
Document code: A
Received date: 2009-06-01

this process[9].

The herbal medicine *Radix Polygonum Multiflorum* has also been reported to be effective in treating AD[10]. Several chemical constituents, particularly the 2,3,5,4'-tetrahydroxy stilbene-2-O-β-glucoside (TSG) identified from the root of *Radix Polygonum Multiflorum,* have been demonstrated to be antioxidant and could delay the ageing effects[11].

The present study aims to observe the hippocampal APP expression induced by chronic Al treatment in rats and the effect of TSG on it. Vitamin E (VE), a powerful biological antioxidant, is effective in improving the cognitive performance of aged rats and in inhibiting formation of senile plaque deposits caused by Al-induced oxidative stress[12]. Thus VE was used as a positive control here.

2 Materials and methods

2.1 Animals A total number of 160 male Sprague-Dawley rats (220-250 g), provided by Experimental Animal Center, Changsha, Hunan, were used in this study. Animals were housed in stainless steel cages under a 12 h light/dark cycle at 22 ºC, with free access to food and water. Animals were allowed to acclimate for at least 10 d prior to the experiment. All the experiments were conducted in accordance with the regulations of Central South University for the use of experimental animals in research, and in conformity with the NIH Guide for the Care and Use of Laboratory Animals.

2.2 Drug preparation TSG was obtained from Hunan Academy of Traditional Chinese Medicine. The extractive procedure from *Radix Polygonum Multiflorum* was conducted as described previously[10]. Briefly, the dried root of *Radix Polygonum Multiflorum* (16 kg) was roughly grounded and refluxed with ethanol twice (each lasted for 1 h). The ethanol extracts were filtered and lyophilized to form powder TSG[14]. For oral administration, the extractive powder of TSG and VE were dissolved in the vehicle vegetable oil at a concentration of 4 g/mL and 40 mg/mL, respectively.

2.3 Passive avoidance task The apparatus of passive avoidance task consisted of an illuminated chamber (20 cm×25 cm×30 cm) and a dark chamber (40 cm×25 cm×30 cm) with a metal grid floor. The 2 chambers were separated by a wall with a guillotine door (8 cm in diameter). The test was performed on 2 consecutive days. In the acquisition trial, rats were placed individually in the illuminated chamber. Once entering the dark chamber, the rat would receive an electric shock (40 V, 0.5 mA, 1 s) on the feet through the floor grid. Then they were immediately removed from the apparatus, back to its home cage. At the retention trial which was conducted 24 h later, the rat was placed again in the illuminated chamber, and the interval between the placement in the illuminated chamber and the entry into the dark chamber (step-through latency) was recorded. If the animal did not enter the dark chamber within the 5-min test period, the test was terminated and the step-through latency was recorded as 300s. Each rat was tested at the end of months 1, 3 and/or 5 of drug treatment. Acquisition and retention trials were included in each test because the avoidance response would disappear on day 26 after acquisition trial[13].

2.4 Experimental design Normal controls (n = 18) and Al-treated rats (n = 70) received oral administration of distilled water and 0.3% aluminum chloride ($AlCl_3$) solution prepared in distilled water, respectively, for 90 d. Although a suppression of thirst desire was observed, which might be due to the change in pH value caused by $AlCl_3$, the rate of water consumption was not affected significantly. All the rats were tested in the passive avoidance task. Al-exposed rats whose step-through latency was decreased by over 60% compared to the average value of the controls were selected (60 out of 70), among which 6 rats were used for Western blot. The rest 54 rats were randomized into vehicle, TSG (4 g/kg) and VE (40 mg/kg) groups. At the end of months 1, 3 and 5 of treatment, the rats were tested in the avoidance task 1 h after vehicle or drug administration, after which they were sacrificed in a batch (n = 6) for APP detection by Western blot. Normal controls did not receive any treatment, but they were also tested in the passive avoidance task and sacrificed in a batch (n = 6) for APP Western blot in the same manner.

2.5 APP Western blot Proteins of the subcellular fractions were extracted as described previously. The hippocampal tissue (approximately 50 mg) was gently homogenized using a Teflon homogenizer (Thomas, Philadelphia, PA), in 7 volumes of cold suspension buffer, containing 20 mmol/L Hepes-KOH (pH 7.5), 250 mmol/L sucrose, 10 mmol/L KCl,

1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1 mmol/L PMSF, 2 mg/mL aprotinin, 10 mg/mL leupeptin, 5 mg/mL pepstatin and 12.5 mg/mL N-acetyl-Leu-Leu-Norleu-Al. The homogenates were centrifuged at 900 g under 4 ºC for 10 min to isolate the nuclear fraction, and then at 8 000 g for 20 min at 4 ºC to separate the mitochondrial fraction from the soluble fraction. The supernatant was further centrifuged at 100 000 g for 60 min under 4 ºC to separate the cytoplasm from the endoplasmic reticulum (ER) fractions. APP, which is reported to be localized in the ER, was examined. Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, IL). Protein (7.5 mg) was separated by 15% SDS-PAGE and transfered to a polyvinylidene difluoride membrane (Millipore, Bedford, MD) at 300 mA for 210 min in transfer buffer (containing 20 mmol/L Tris-base, 150 mmol/L glycine, and 20% methanol). After that, membranes were incubated overnight at 37 ºC with mouse anti-APP at a 1:250 dilution. β-Actin served as a loading control. Proteins were visualized with enhanced chemiluminescence (Immun-Star detection kit, Bio-Rad, Hercules, CA). The bands of APP and β-actin were scanned and densitometrically analyzed using Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

2.6 Statistical analysis All the data were expressed as mean±SEM. Two-way analysis of variance (ANOVA) was used to evaluate group×duration of treatment interaction, followed by post hoc multiple comparisons using Student-Newman-Keuls test to further analyze the differences between groups. $P < 0.05$ was considered as significantly different.

3 Results

3.1 Passive avoidance task Fifty-four out of 60 Al-exposed treated rats showed a decrease (>60%) in the step-through latency, and their average value was (50.2±8.8) s, only 19% of the control value (267.8±7.2) s. The time course of attenuation of the step-through latency by TSG and VE in Al-treated rats was shown in Fig. 1. Two-way ANOVA analysis revealed a significant interaction between group and duration of treatment on the step-through latency ($P < 0.05$). Multiple comparisons further showed that at the 3- and 5-month points, the latency in TSG group was significantly extended compared to that in vehicle group ($P < 0.05$). Furthermore, the extended latency of TSG group was markedly greater than that of VE group at the 5-month time point ($P < 0.05$).

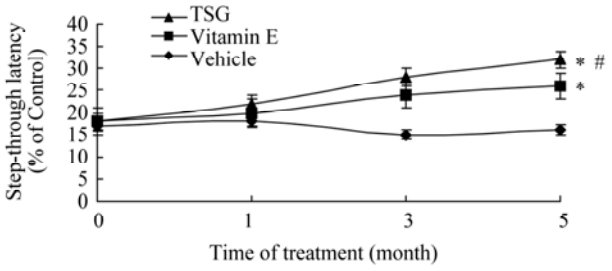

Fig. 1 The reversal effects of long-term treatment with TSG and VE against the decrease of step-through latency in Al-exposed rats. The rats were given orally vehicle, TSG (4 g/kg) or VE (40 mg/kg) for up to 5 months, and tested in the passive avoidance task at the end of 1, 3 and 5 months of treatment. Data were expressed as percentage of time-matched normal controls. *$P < 0.05$ *vs* vehicle group, #$P < 0.05$ *vs* VE group.

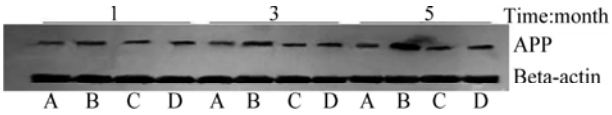

Fig. 2 Western blot analysis of APP protein level in the hippocampus at the end of 1, 3 and 5 months of treatment. A: control group; B: vehicle group; C: TSG group; D: VE group.

3.2 APP Western blot analysis As shown in Fig. 2, APP (124 kDa) was detected in the ER fraction in vehicle, VE and TSG groups. A very faint band corresponding to APP could be seen in the control group. In comparison, Al treatment in-

Table 1. Densitometric analysis of APP protein level in control, vehicle, TSG and VE groups

Group	*n*	1 month	3 month	5 month
Control	6	0.77±0.21	0.80±0.11	0.78±0.13
Vehicle	6	1.72±0.09*	1.86±0.08*	2.21±0.23*
TSG	6	1.65±0.18*	1.52±0.13*▲★	1.45±0.12*▲★
VE	6	1.68±0.14*	1.78±0.10*	1.70±0.21*▲

*$P < 0.05$ *vs* control group, ▲$P < 0.05$ *vs* time-matched vehicle group, ★$P < 0.05$ *vs* time-matched VE group.

duced a significant increase in APP protein level ($P < 0.05$), and both VE and TSG could inhibit this increase. Multiple comparisons further revealed a significant decrease in the expression of APP in TSG group at the end of months 3 and 5 ($P < 0.05$), and in VE group at the end of month 5, as compared to that in vehicle group at the matched time point ($P < 0.05$, Table 1).

4 Discussion

Although the pathogenesis of AD has not yet been fully understood, several hypotheses have been established to explain the multifactorial characteristic of this disease. Among the relevant factors, metal ions could aggravate the course of AD[14]. Al ion exposure could cause conformational change of Aβ[15,16]. Besides, Al could cause the accumulations of tau protein and Aβ in experimental animals, inducing apoptosis *in vivo* and *in vitro*[17] and altering enzymatic degradation of Aβ[18]. Al could also alter the concentrations of amino acid neurotransmitters, their metabolites, and the activities of neuropeptides cholecystokinin (CCK) and substance P, in several brain regions[29-21]. Intraventricular microinjection of Al has been reported to induce inflammatory responses of neuroglia[22], which raises the possibility that Al may aggravate the neurodegenerative disease. The neurotoxicity of Al is likely to be the result of a combination of several mechanisms, including oxidative brain injury, amyloid deposits and neuroglia inflammatory reaction, and reduced neurotransmitter biosynthesis.

APP expression and processing in the brain is a critical event in the development of AD[23,24]. Here, chronic exposure to Al reduced the latency of avoidance response and increased the expression of APP. Since a high level of APP leads to increased Aβ production, which facilitates Aβ deposition in the hippocampus, we propose that Al may be a potential contributor for neuritic plaque formation and cognitive impairment in Alzheimer's disease.

Here, we further find that the Al-induced cognitive impairment could be ameliorated by chronic VE treatment. Besides, VE can suppress the overexpression of hippocampal APP in Al-treated rats. Since VE has the ability to reduce oxidative stress and inhibit amyloid deposits[25], the present results suggest that Al-induced cognitive and neurodegenerative changes are directly related to the induced oxidative brain injury and amyloid deposits. Moreover, the principal finding of the present study is that long-term treatment with TSG can attenuate Al-induced cognitive impairment, overexpression of hippocampal APP, and Aβ deposit. These effects of TSG are strengthened with the time of treatment, and exceed those of the antioxidant agent VE after 5 months of treatment.

Based on the chemical constituent elements of TSG, there are 3 possible mechanisms by which TSG protects the brain against the neurotoxic effects of Al. Stilbene has been identified to be the most abundant bioactive component in the root of *Polygoni Multiflori*[26], and 2,3,5,4'-tetrahydroxy stilbene-2-O-β-glucoside (TSG) has been found to possess the anti-nociceptive property, which is mediated by multiple receptor systems, including GABA, glutamate, noradrenaline, serotonin and neuropeptides[27], suggesting that TSG may have the capacity of inhibiting the interfering effects of Al on neurotransmitter biosynthesis. Finally, although little is known about the mechanisms underlying the anti-inflammatory actions of *Polygoni Multiflori*, clinical observations have shown its effectiveness in treating rheumatic illness[28]. *Polygoni Multiflori* extract TSG may have similar effects against the Al-induced inflammatory response of neuroglia cells.

In summary, the present study demonstrates that long-term treatment with TSG significantly ameliorates the cognitive impairment in the passive avoidance task in Al-treated rats. TSG also suppresses the Al-induced over-expression of hippocampal APP. The protective effects of TSG against Al neurotoxicity are significantly greater than those of the antioxidant agent VE, suggesting that the protective properties of TSG may derive from multiple mechanisms, including the antioxidant effect. It also indicates that in Alzheimer's disease, Al can raise the expression of APP, which in turn, causes Aβ overproduction and deposition in hippocampus, one of the triggers for learning and memory disturbance in rat models. The potential relevance of the present results to clinical therapeutics in the treatment of Alzheimer's disease needs further investigations.

Acknowledgement: The work was supported by the Natural Science Foundation of Hunan Province, China (No. 20227).

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二苯乙烯苷改善铝诱导的认知损害并减少β-淀粉样前体蛋白的表达

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摘要：目的 过量的铝盐可损害人和动物的神经认知功能。流行病学研究提示，慢性铝中毒和阿尔茨海默病有潜在的联系。本实验旨在评估中药何首乌提取物——二苯乙烯苷(TSG)对铝盐慢性中毒的大鼠认知功能的保护作用及海马部位β-淀粉样前体蛋白(APP)表达的影响。**方法** 以水配制0.3%氯化铝，连续喂养大鼠90天。随后铝盐暴露的大鼠随机分组，采用溶媒，进行TSG (4 g/kg)和VE (40 mg/kg)干预5个月，其中VE作为对照药物。结束后进行电刺激实验，并用Western blot法检测APP的蛋白水平。**结果** 连续服用90天$AlCl_3$后，大鼠躲避电刺激能力显著下降，并且海马部位APP的表达显著上调。经过TSG和VE干预后，大鼠躲避电刺激能力得到显著改善，APP表达的上升也被显著抑制。与VE相比，TSG的效果随时间延长而变得更为显著。**结论** TSG对阿尔茨海默病有一定的治疗作用。

关键词：二苯乙烯苷；认知损害；β-淀粉样前体蛋白；铝盐