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Proanthocyanidins affects the neurotoxicity of Aβ25-35 on C57/bl6 mice

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Abstract. – OBJECTIVE: To investigate the influence of procyanidins on the impairment of memory.

MATERIALS AND METHODS: Thirty male C57/bl6 mice were divided into five groups: low, middle, and high concentration, model, and control groups. Intracerebroventricular injection of β-amyloid25-35 in C57/bl6 mice caused an impairment of learning and memory. Next day, intragastric administration of procyanidins in the treatment group mice: (lower, middle and high concentration). Hoechst staining observed apoptosis of neuronal nuclei in the hippocampus. Immunohistochemistry determined synaptic remodeling reaction and the expression level of glial inflammatory response.

RESULTS: Compared with the model group, the proportion of neuronal apoptosis decreased in the hippocampal CA1 region of the treatment group. The Synaptic (SYN) density was increased, and the level of activated astrocytes and microglia expression in the hippocampus was decreased.

CONCLUSIONS: Procyanidins have a protective influence on Aβ25-35 mice hippocampus neuron, reducing nerve cell damage and eases learning and memory deficit.

Key Words:
Alzheimer s disease, Amyloid beta-protein, Procyanidins, Gliosis, Oxidative stress.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease. The world has more than 35,000,000 people suffering from Alzheimer's disease, which occurs in the elderly[1]. Alzheimer's disease patients had degradation of memory and other cognitive areas and a medium survival rate of 3 to 9 years after diagnosis. Deposition of Aβ plaques and neurofibrillary tangles accumulated by hyper-phosphorylated tau protein are the major pathological features of AD[2]. However, the causes of AD are, as yet, unknown. Of all the etiological hypothesis, Aβ plaques are the determinant of AD[3]. There are no effective prevention and treatment measures of AD. For now, all treatment is limited to delay the onset and progression of the disease. Proanthocyanidins are polyphenolic compounds have the protective effects of being antioxidants, anti-tumor, anti-inflammatory, anti-mutation, and antiviral[4]. In our study, we detected the mice hippocampus inflammatory, the apoptosis of neurons and synaptic remodeling, responding to learning and memory impairment induced by Aβ25-35 in mice. This provides a more theoretical basis for procyanidins treatment of Alzheimer's disease.

Materials and Methods

For the experiment, we used the following**:** 30 two-month old male C57/bl6 clean level mice, weight (22.5 ± 1.5) g (provided by Vital River Laboratory Animal Technology Co. Ltd. Nanjing branch), Aβ25-35 (Sigma, St. Louis, MA, USA), Pine Proanthocyanidin (purity > 95%, purchased from Nanjing University of Chinese Medicine), Hoechst 33342 (Vector Laboratories, Burlingame, CA, USA), SYN (Millipore, Billerica, MA, USA), glial fibrillary acidic protein (GFAP, DAKO, Carpinteria, MA, USA), Anti-Iba-1 (Ionized calcium binding adaptor molecule-1 (Iba1, Abcam, Cambridge, MA, USA), blocked by goat serum, IgG-HRP (ZSJQ-Bio Co. Ltd.), DAB Horseradish Peroxidase Color Development Kit (Sigma, USA), stereotaxic apparatus (BWSR-5M, Narishige, Tokyo, Japan). Paraffin series are of Leica Company (Wetzlar,Germany), Upright Metallurgical Microscope and image acquisition system of Japanese Olympic company products (Tokyo, Japan).

Thirty male C57/bl6 mice were divided into five groups randomly: 1) low concentration group (intracerebroventricular injection (i.c.v.) Aβ25-35 + 50 mg/kg proanthocyanidin 10 ml/kg

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by gavage); 2) middle concentration group (i.c.v Aβ25-35 + 100 mg/kg proanthocyanidin 10 ml/kg by gavage); 3) high concentration group (i.c.v Aβ25-35 + 150 mg/kg proanthocyanidin 10 ml/kg by gavage); 4) model group (i.c.v Aβ25-35 + sterile double distilled water 10 ml/kg by gavage); 5) control group (i.c.v saline + Sterile double distilled water 10 ml/kg by gavage). All mice were housed at 20-25°C, 60% relative humidity at Nanjing Medical University Experimental Animal Center.

For the animal models[5], we used 1 ml sterile saline to dissolve 1 mg Aβ25-35, then packaged and preserved them at -20°C, for 7 days of gatheration at 37°C in a water bath before the surgery. The mice were anesthetized with chloral hydrate (3%) and positioned on a stereotaxic instrument. Aβ 25-35 was injected into each lateral ventricle using a micro syringe for 10 min. The coordinates were as follows: –0.22 mm posterior, 1.0 mm lateral and +2.55 mm ventral under Paxinos and Franklin. The micro syringe was withdrawn five minutes after the end of injection. The control and treatment mice received an injection of the same volume of saline. All procedures were performed under aseptic conditions.

All mice's brains were prepared after gastric lavage by separating the left and right sides. The right forebrain was dehydrated and embedded to make paraffin slices.

Hoechst Staining Method

Cell apoptosis is characterized by an increased membrane permeability and karyopyknosis. Therefore, Hoechst staining of the apoptotic cells is brighter compared to normal cells. The slices of -2.18 mm level were stained with Hoechst. We de-waxed and hydrated the paraffin sections, adding Hoechst (1:500), then incubated at 37°C for 20 min, washed with phosphate buffered saline (PBS) for 3 times (5 min each time), sealed with fluorescence bleacher. According to the double-blind principle, we calculated the percentage of Hoechst positive cell using the Image-ProPlus 6 image analysis software on the brain slices of hippocampus CA1 region (* 200). Each mouse received a mean respectively and was compared to the control group for statistical analysis.

Immunohistochemistry

Synaptic (SYN) immunohistochemistry was performed on the slices of 2.08 mm level posterior from bregma. Glial fibrillary acidic protein (GFAP) staining of the astrocytes and the Iba1 staining of microglial cells in the slices of 2.28 and 1.98 level respectively. The slices were repaired by microwave, and incubated SYN1:1000, GFAP1:1000, Iba1 1:800 at 4°C overnight, then incubated HRP-antigen (1:200) at 37°C for 1h and visualized using diaminobenzidine (DAB). We assessed the expression level of SYN, GFAP and Iba1 in the hippocampus using Image-Pro-Plus 6 image analysis software (Bethesda, MD, USA).

Statistical Analysis

The statistical method – we used the SPSS21.0 statistic software (SPSS Inc., Chicago, IL, USA) for the analysis. The *t*-test was used to compare two groups. ANOVA was used to compare multiple groups. Values were expressed as $p<0.05$; the differences were statically significant.

Result

Hoechst Staining Results

The neurons of the mice hippocampus CA1 area in the model group was arranged in disorder. The mount of hyper-chromatic nuclei nerve cells increased significantly compared with the control group (4.03±0.26, 0.85±0.20, t=9.816, p=0.000)**.** The number of hyper-chromatic nuclei nerve cells was significantly reduced in all kinds of the PC treatment groups compared with the model group (3.72±0.90, 2.47±0.12, 1.03±0.17, 4.03±0.26, F=4.45, p=0.019), in a dose-dependent manner (Figure 1, Table I). It shows that Aβ25-35 can induce the apoptosis of hippocampus neurons in mice, and procyanidins can significantly reduce its neurotoxicity.

SYN Staining Results

The SYN density in the hippocampal CA1 area of the mice in the model group decreased significantly compared with the control group (0.0785±0.0009, 0.0848±0.0007, t=-5.753, p=0.005). The SYN density in the hippocampal was reduced in all PC treatment groups compared with the model group (0.0935±0.0025, 0.0862±0.0010, 0.0802±0.0029, 0.0785±0.0009, F=9.413, p=0.004). The SYN density increased in the high concentration group compared with the model group, but there was no statistically significant difference (Figure 2, Table II).

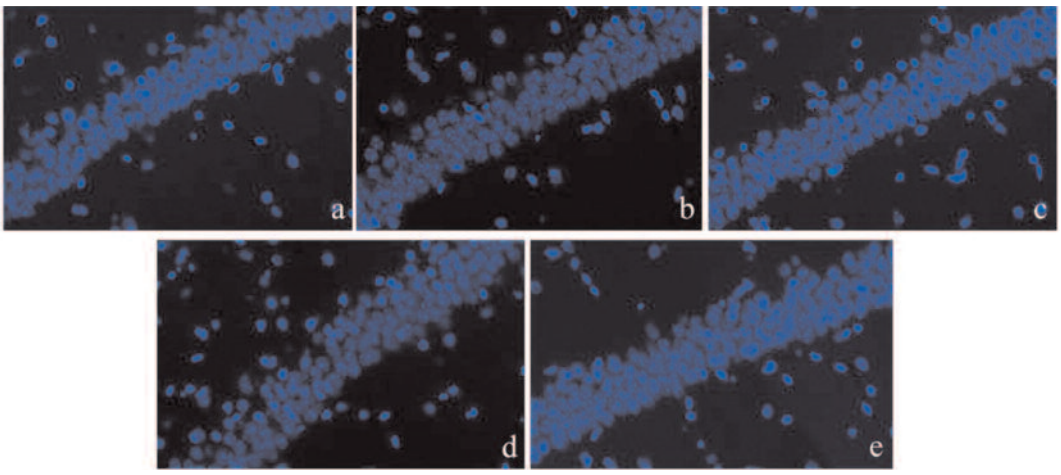

Figure 1. Cell apoptosis was determined by Hochest staining. ***(a)*** Low concentration group, ***(b)*** Middle concentration group, ***(c)*** High concentration group, ***(d)*** model grou, ***(e)*** Control group.

Glial Activation Reaction Results

The number of cells activated by Iba1 and the GFAP density in the mice hippocampus increased in model group compared with the control group (Average optical density of GFAP: 0.1127±0.0017, 0.1015±0.0018, t=4.459, p=0.011; Iba1 counting: 8.14±0.75, 4.64±0.20, t=4.48, p=0.002). The number of cells activated by Iba1 and the GFAP density in the hippocampus was reduced in all PC treatment groups compared with the model group (average optical density of GFAP: 0.1035±0.0017, 0.0993±0.0029, 0.1001±0.0023, 0.1127±0.0017, F=5.484, p=0.015; Iba1 counting: 8.98±0.26, 5.81±0.24, 3.74±0.69, 8.14±0.75, F=20.795, p=0.000) (Figures 3, 4, Table III).

Discussion

Alzheimer's disease is a progressive, irreversible neurodegenerative disease, which is the most common type of dementia and accounts for about 60%-70%. The prevalence increases with age. The incidence rate of 65-74 years old people was 3% and people above the age of 85 rose to 50%[6]. Alzheimer's disease is an acquired syndrome. Its main characteristics are decreased or loss of memory and other cognitive abilities. The course of AD is usually 7 to 10 years, leading to death from various complications[7]. Most AD patients are sporadic, and the proportion of a genetic type is less than 1%. Age is an important risk factor of AD. Plaques deposits and neurofibrillary tangles with age[8].

Aβ are small molecular fragments of amyloid precursor protein (APP) digested by intracellular β and γ secretase. Abnormal accumulation and precipitation of Aβ in the brain will result in neurotoxicity. Although the etiology of AD is still not unclear, the Aβ toxicity cascade is recognized as the key to the pathogenesis of it[9]. Oxidative stress and glial inflammatory reaction are factors that play a crucial role in the pathophysiology of Aβ. The central link to oxidative stress damage is mass production of oxygen free radicals. Increasing evidence suggests that the oxidative stress may be the initiation factor of AD[10]. An imbal-

Table I. Comparison of Hochest positive cells in the hippocampal C1 region.

Group	cases	The percentage of apoptosis nerve cell in CA1
Low concentration group (a)	6	3.72 ± 0.90
Middle concentration group (b)	6	2.47 ± 0.122
High concentration group (c)	6	1.03 ± 0.172
Model group (d)	6	4.03 ± 0.261
Control group (e)	6	0.85 ± 0.20

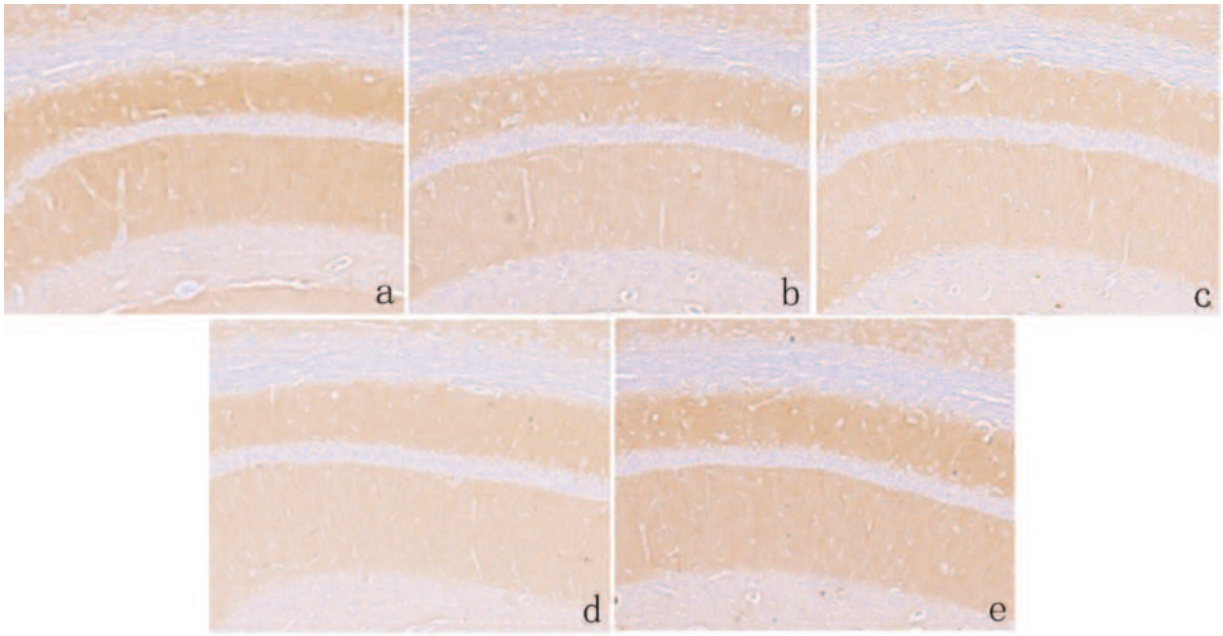

Figure 2. SYN immunohistochemistry staining. ***(a)*** Low concentration group, ***(b)*** Middle concentration group, ***(c)*** High concentration group, ***(d)*** model grou, ***(e)*** Control group.

Table II. Comparison of SYN density in hippocampal C1 region.

Group	Cases	SYN density
Low concentration group a	6	0.0935 ± 0.00252
Middle concentration group b	6	0.0862 ± 0.00192
High concentration group c	6	0.0802 ± 0.0029
Model group d	6	0.0785 ± 0.00091
Control Group e	6	0.0848 ± 0.0007

Note: Compared with the control group, 1p<0.05; Compared with the model group, 2p<0.05

Table III. Comparison of the level of activated astrocytes and microglia expression.

	Group	Cases	SYN density
Low concentration group a	6	0.1035 ± 0.00172	8.98 ± 0.26
Middle concentration group b	6	0.0993 ± 0.00292	5.81 ± 0.242
High concentration group c	6	0.1001 ± 0.00232	3.74 ± 0.692
Model group d	6	0.1127 ± 0.00171	8.14 ± 0.751
Control Group e	6	0.1015 ± 0.0018	4.64 ± 0.20

Note: Compared with the control group, 1p<0.05; Compared with the model group, 2p<0.05

ance of pro-oxidant and antioxidant homeostasis in the body can lead to toxic reactive oxygen species (ROS) production, resulting in the generation of Aβ[11]. The aggregation of Aβ in the brain, astrocytes and microglia activation and synapses damage causing oxidative stress injury, leading to cell apoptosis[12]. Astrocytes are the most abundant non-neuronal cells in the central nervous system. They can regulate the extracellular ion and energy storage, the clearance and metabolism of the neurotransmitter, and maintain normal neuronal function in the central nervous system. As well, it can activate Aβ and release cytokines which are caused by the production of Aβ. The pro-inflam-

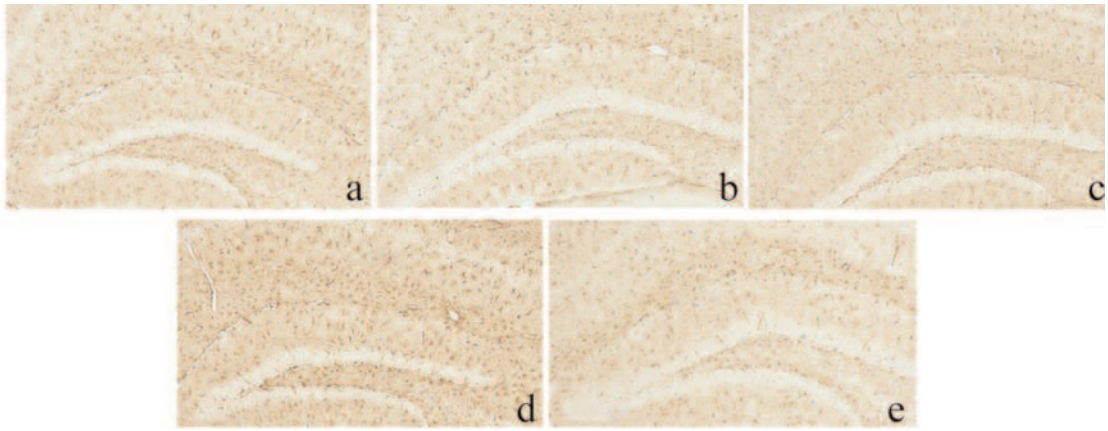

Figure 3. GFAP staining of the astrocytes. ***(a)*** Low concentration group, ***(b)*** Middle concentration group, ***(c)*** High concentration group, ***(d)*** Model grou, ***(e)*** Control group.

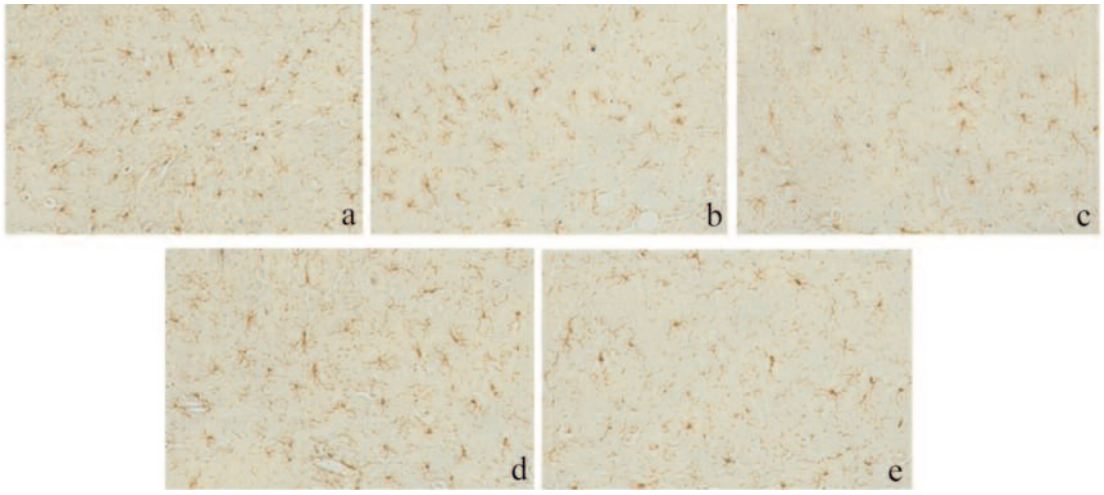

Figure 4. Iba1 staining of microglial cells. ***(a)*** Low concentration group, ***(b)*** Middle concentration group, ***(c)*** High concentration group, ***(d)*** model grou, ***(e)*** Control group.

matory molecules secreted by activated astrocytes can increase the expression of the neuron endocrine enzyme, Aβ production, and further activate microglia to produce the pro-inflammatory factor at the same time[13]. This research adopts the learning and memory impairment in mice model induced by intracerebroventricular injection of Aβ25-35. The results suggest that the glial inflammatory reaction in the hippocampus of the mode group increased significantly compared with the control group. These findings are consistent with former research studies.

Nowadays, due to an aging population, patients with AD are ever increasing, and there is still no effective treatment[14]. At present, drugs for the clinical treatment of AD improve clinical symptoms of AD mainly through the inhibition of Ach E and increasing the Ach in patients. Methods used are: to increase the synthesis and release of Ach, inhibit the degradation of Ach and Ach receptor agonists. However, because of its serious side effects more research on the treatment of AD is still on going. In recent years, more and more evidence showed that oxidative stress and inflammation plays a significant role in the pathogenesis of AD. Therefore, many researchers are using antioxidant and anti-inflammatory treatments to slow the progression of the disease[15]. Proanthocyanidin is a potent antioxidant which is widely distributed in many plants[16]. Now the PC is used in many fields, such as cardiovascular, anti-tumor, kidney disease. It has obtained positive results[17]. PC can reduce free radicals generation, thus, inhibiting lipid peroxida-

tion, and reducing the astrocytes and microglia activation[18]. Many studies have confirmed the significant efficacy of antioxidants in the treatment of AD[19]. In this study, based on its antioxidant effect, we use different concentrations of the pine bark extract PC by gastric perfusion on mice model induces by Aβ 25-35 and, then, observe the effects of various concentrations of PC on learning and memory function in the mice. We found that high and middle concentrations of PC can decrease the apoptosis in the hippocampal neuron of the AD mice model. The low and middle concentration of PC increased the density of hippocampal SYN significantly. Reduced active cells of GFAP and Iba1 were observed in all three model groups. Speculation is that proanthocyanidin can reduce astrocytes and microglia activation degree based on its antioxidant effect, reducing the Aβ aggregation, which in turn may reduce oxidative stress injury, so as to improve the learning and memory in AD mice, thus, delaying disease progression.

Conclusions

In this study, we found that proanthocyanidins have a protective effect on Aβ25-35 mice. It can significantly improve learning and memory impairment in the Aβ25-35 mice and reduce the glial inflammatory reaction caused by Aβ25-35 toxicity, providing more evidence for PC in treating AD. However, the mechanism of PC in the treatment of AD is still unclear, so further research is needed on the molecular mechanism of antioxidant treatment of procyanidins in order to provide a more theoretical basis for therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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