Effects of Honeysuckle Chlorogenic Acid on Secretory Enzymes, Lipoxygenase A4, and Biochemical Indicators in Model Mice with Aluminum Induced Alzheimer's Disease

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Abstract [**Objectives**] To explore the effects of chlorogenic acid from honeysuckle on the secretion enzymes, lipoxygenase A4 (LXA4), and blood biochemical indicators in mice with aluminum induced Alzheimer's disease (AD). [**Methods**] Chlorogenic acid was extracted from honeysuckle by ultrasound assisted alcohol extraction method. Seventy mice were randomly divided into normal group, model group, and low, medium and high dose groups of honeysuckle chlorogenic acid. All the mice in each group except for the normal group were given maltol aluminum by intraperitoneal injection to establish models of aluminum induced AD, continuously injected for 5 d and stopped for 2 d, totally poisoned for 8 weeks. Starting from the 5th week of poisoning, the low, medium and high dose groups of honeysuckle chlorogenic acid were given honeysuckle chlorogenic acid solution 40, 80 and 160 mg/kg by gavage, respectively, while the normal group and the model group were fed with an equal volume of distilled water, all once daily, continuously gavaged until the end of the 8th week. At the end of the experiment, the learning memory ability of the mice was tested by Y-type water maze, and the number of tests required to reach the learning standard, the number of memory errors in 20 tests and the error rate of the mice were recorded. The brains of mice were taken to determine the contents of β-secretase, α-secretase, γ-secretase, LXA4 and acetylcholinesterase (AchE) in the homogenates of brain tissues by ELISA, and their blood was taken to determine the biochemical indexes. [**Results**] Compared with the normal group, the number of learning tests, number of memory errors, error rate and the contents of β-secretase, γ-secretase and AchE in brain tissue of the mice in the model group were all significantly increased (all $P<0.05$), the contents of LXA4 in brain tissue were significantly decreased (all $P<0.05$), and the contents of α-secretase did not change significantly (all $P>0.05$); compared with the model group, the number of learning tests, the number of memory errors, the error rate and the content of β-secretase, γ-secretase and AchE in brain tissue were all significantly reduced (all $P<0.05$), the content of LXA4 in brain tissue of the high dose group of honeysuckle chlorogenic acid was significantly increased ($P<0.05$), and there was no significant change in the content of α-secretase in brain tissue of all groups of honeysuckle chlorogenic acid (all $P>0.05$). Compared with the normal group, the levels of blood glucose, TC, TG, ALT, BUN, Cr and UA in the model group and the levels of TC, TG and BUN in the low- and medium-dose groups of honeysuckle chlorogenic acid were significantly increased (all $P<0.05$), and the level of HDL-C in the model group and the levels of UA in the medium-and high-dose groups of honeysuckle chlorogenic acid were significantly decreased (all $P<0.05$); compared with the model group, the levels of blood glucose, ALT, BUN, UA in each group of honeysuckle chlorogenic acid, the levels of TC and Cr in medium and high dose groups of honeysuckle chlorogenic acid, and the level of TG in the high dose group of honeysuckle chlorogenic acid were all significantly lower (all $P<0.05$), while the level of HDL-C in the medium and high dose groups of honeysuckle chlorogenic acid and the level of total protein in the high dose group of honeysuckle chlorogenic acid were all significantly higher (all $P<0.05$). [**Conclusions**] Chlorogenic acid from honeysuckle may improve AD induced by aluminum exposure via regulating related secretory enzymes, LXA4, and various biochemical indicators.

Key words Alzheimer's disease, Chlorogenic acid, Secretase, Lipoxygenase A4

1 Introduction

The pathogenesis of Alzheimer's disease (AD) is complex, and the beta amyloid (Aβ) cascade theory is currently one of the most widely recognized mechanisms. This theory suggests that Aβ aggregation in the brain is a primary change in AD patients, and other pathological processes are caused by an imbalance in the production and clearance of Aβ. Among them, β-secretase plays an important role, and it can limit the production of Aβ by inhibiting β-secretase. Therefore, finding substances that inhibit β-secretase activity is of great significance for the prevention and treatment of AD[1]. Aluminum is a chronic neurotoxin that is abundant in neurofibrillary tangles (NFT) of the brain and can cause AD[2]. At present, there are no curative drugs for AD in clinical practice, and traditional Chinese medicine is increasingly being valued in the treatment of AD due to its low incidence of adverse reactions and low cost. This study used ultrasound assisted alcohol extraction to extract chlorogenic acid from honeysuckle, and established an aluminum toxicity induced AD mouse model by intraperitoneal injection of maltol aluminum solution. The effects of chlorogenic acid on learning and memory, brain related secretion enzymes, lipid oxygen A4 (LXA4), and blood biochemical indicators in the poisoned mice were observed to elucidate the mechanism of effective ingredients in traditional Chinese medicine

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intervening in AD formation and develop effective anti AD drugs.

2 Materials and methods

2.1 Experimental animals 70 SPF-grade mice (38 females and 32 males) weighing 25 – 30 g were purchased from Changsha Tianqin Biotechnology Co., Ltd. with production license No. of SCXK (Xiang) 2022-0011. They were fed in independent rat cage feeding boxes (Guangdong Zhuhai Zaixin Instrument Co., Ltd.), 3 – 5 mice each cage. They were numbered, and male and female were separated, free to drink tap water. Air was filtrated and purified, and room temperature was controlled at 26 – 28 ℃ by air conditioning. They adapted to the environment for 7 d. Experiments were conducted after applying for experimental animal use and ethical review.

2.2 Drugs Dried honeysuckle products (commercially available, Baise City, Guangxi) were dried in a 65 ℃ constant temperature oven until constant weight was reached. After crushed, it passed through a screen of 30 – 120 meshes and was prepared into air dried samples. According to the method in reference[3], chlorogenic acid was extracted, and its content was determined as 3.6 mg/mL. Chlorogenic acid standard sample (Shanghai Yuanye Biotechnology Co., Ltd.).

2.3 Main reagents and instruments β-secretase, α-secretase, γ-secretase, LXA4, acetylcholinesterase (AchE), total cholesterol (TC), triacylglycerol (TG), high-density lipoprotein cholesterol (HDL-C), blood sugar, alanine aminotransferase (ALT), total protein, urea nitrogen (BUN), uric acid (UA), creatinine (Cr) test box and crystalline aluminum chloride ($AlCl_3 \cdot 6H_2O$), sodium chloride, maltol, anhydrous ethanol (AR), normal saline (Guangxi Nanning Juyuan Instrument Co., Ltd.), 0.01 mol/L of phosphate buffer solution (PBS) with pH 7.4; SpectraMax M5 type of enzyme-linked immunosorbent assay (ELISA) reader; Y-shaped water maze stimulator, UV-5500 type of UV visible spectrophotometer (Shanghai Yuanxi Scientific Instrument Co., Ltd.), FB224 type of electronic balance (Shanghai Shunyu Hengping Scientific Instrument Co., Ltd.), FZ102 type of plant crusher (Tianjin Taisite Instrument Co., Ltd.), N-1100S-WD type of rotary evaporator (Tokyo Physical and Chemical Institute, Japan), SM900A type of ultrasonic cell grinder (Nanjing Shunma Instrument Equipment Co., Ltd.), DS-5510DTH type of ultrasonic cleaning machine (Shanghai Shengxi Ultrasonic Instrument Co., Ltd.), Master touch 602 type of ultra pure water analyzer (Shanghai Hetai), DGT-G135S type of electric hot blast drying oven (Hefei Daskat Biotechnology Co., Ltd.), TG16-WS type of high-speed desktop centrifuge (Hunan Xiangyi), HH-42 type of digital display electric constant-temperature water temperature box (Shanghai Yuejin).

2.4 Grouping, modeling, and intervention The mice were randomly divided into a normal group, a model group, and low, medium, and high dose groups of honeysuckle chlorogenic acid, with 14 mice in each group. Except for the normal group, all other groups of mice were given an intraperitoneal injection of 0.8 mg/(kg · d) of maltol aluminum solution (mixed with equal volumes of maltol solution and aluminum trichloride solution, adjusted pH to 7.1 – 7.4, filtered and used) for 8 weeks. They were injected continuously for 5 d and intermittently for 2 d. Starting from the 5th week of infection, the low, medium, and high dose groups of honeysuckle chlorogenic acid were given honeysuckle chlorogenic acid solution at doses of 40, 80, and 160 mg/kg (calculated based on the extracted chlorogenic acid content of honeysuckle at 3.6 mg/mL) by gavage, respectively. The normal group and model group were given equal volumes of distilled water once a day until the end of the experiment. During the experiment, all mice were fed with regular feed, free access to water and food, natural circadian lighting, and no aluminum containing products were used for cages, water bottles, or other utensils. The behaviors, feces, hair removal, feeding, weight loss, and death of mice were observed and recorded every day.

2.5 Testing indicators and methods

2.5.1 Learning and memory abilities. After the experiment, according to the method described in reference[4], the learning and memory abilities of each group of mice were tested using a Y-shaped water maze stimulator. The number of tests required for each group of mice to reach the learning standard, the number of memory errors in 20 tests, and the error rate were recorded.

2.5.2 Contents of related secretory enzymes and LXA4 in brain tissue. After the completion of the water maze experiment, each group of mice was euthanized, and brain tissue was taken and stored in a −20 ℃ freezer for later use. Before use, the blood stains on the surface of the brain tissue were washed with 0.01 mol/L of PBS, dried with filter paper, weighed, and ground with a glass homogenizer in an ice bath for 10 min to prepare a 10% brain homogenate with PBS. The supernatant was taken and stored in a −20 ℃ freezer. According to the instructions of the enzyme-linked immunosorbent assay kit, the contents of β-secretase, α-secretase, γ-secretase, LXA4, and AchE in the homogenate were measured. Each sample was equipped with corresponding wells, and the test results of all samples were within the standard curve range.

2.5.3 Serum biochemical indicators. Before euthanizing at the end of the experiment, blood was collected from the eyeballs of each group of mice, and serum was separated. According to the instructions in the reagent kit, blood glucose, TC, TG, HDL-C, total protein, ALT, BUN, Cr, UA levels were detected.

2.6 Statistical methods The data was processed using SPSS 17.0 software, and normality and homogeneity of variance tests were performed on the metric data. Data that conforms to normal distribution were represented by $\bar{x} \pm s$, and pairwise comparisons were performed using Q-test. $P < 0.05$ indicated statistically significant differences.

3 Results and analysis

3.1 Survival status and status of mice in each group After the experiment, 13 mice in the normal group, 11 mice in the model group, 12 mice in the low-dose chlorogenic acid group, 11 mice

in the medium-dose chlorogenic acid group, and 12 mice in the high-dose chlorogenic acid group survived. The mice in the model group were relatively thin and had delayed movements, while the other groups showed no abnormal clinical manifestations.

3.2 Learning and memory status of mice in each group The number of learning tests, the number of memory errors, and error rates in the model group of mice were significantly higher than those in the normal group (all $P<0.05$). The number of learning tests, the number of memory errors, and error rates in the honeysuckle chlorogenic acid group of mice were significantly lower than those in the model group (all $P<0.05$). The number of learning tests and the number of memory errors in the high-dose chlorogenic acid group of mice were significantly lower than those in the low-dose chlorogenic acid group (all $P<0.05$). There was no statistically significant difference in error rate among the different dose groups of chlorogenic acid (all $P>0.05$). The results were shown as Table 1.

Table 1 Comparison of learning and memory test results between normal and AD groups of mice

Group	Number	Number of learning tests ($\bar{x}\pm s$, times)	Number of memory errors ($\bar{x}\pm s$, times)	Error rate//%
Normal	13	14.38±1.63	4.87±1.34	3.86
Model	11	20.57±2.64①	10.27±2.53①	8.89①
L	12	18.88±2.87②	7.52±1.42②	6.78②
M	11	16.23±1.33②	5.14±1.09②	6.29②
H	12	15.04±1.26②③	4.21±1.11②③	5.31②

NOTE L, M, and H are low-, medium-, and high-dose groups of honeysuckle chlorogenic acid, respectively. ① Compared with normal group, $P<0.05$; ② Compared with model group, $P<0.05$; ③ Compared with low-dose group of honeysuckle chlorogenic acid, $P<0.05$. The same below.

3.3 Comparison of β-secretase, α-secretase, γ-secretase, LXA4, AchE content in brain tissues of mice in different groups The levels of β-secretase in both the model group and the honeysuckle chlorogenic acid group were significantly higher than that in the normal group (all $P<0.05$), and the levels of β-secretase in all groups of honeysuckle chlorogenic acid were significantly lower than that in the model group (all $P<0.05$). There was no statistically significant difference in the content of α-secretase among the groups (all $P>0.05$). The levels of γ-secretase and AchE in the model group were significantly higher than those in the normal group (all $P<0.05$), while the levels of γ-secretase and AchE in the honeysuckle chlorogenic acid groups were significantly lower than those in the model group (all $P<0.05$). The LXA4 content in the model group and the low- and medium-dose groups of honeysuckle chlorogenic acid were significantly lower than that in the normal group (all $P<0.05$), while the LXA4 content in the high-dose group of honeysuckle chlorogenic acid was significantly higher than that in the model group ($P<0.05$). There was no statistically significant difference in the contents of β-secretase, γ-secretase, LXA4, and AchE among the groups of honeysuckle chlorogenic acid (all $P>0.05$). The results were shown as Table 2.

Table 2 Comparison of β-secretase, α-secretase, γ-secretase, LXA4, AchE content in mice brain tissues of normal and AD groups ($\bar{x}\pm s$)

Group	Number	β-secretase IU/L	α-secretase IU/L	γ-secretase//IU/L	LXA4//ng/mL	AchE//IU/mg Pro
Normal	13	24.82±5.57	22.94±2.25	11.88±1.60	14.55±4.10	0.69±0.24
Model	11	34.78±2.59①	25.05±1.40	15.03±2.37①	7.96±5.33①	0.89±0.10①
L	12	28.48±2.02①②	23.01±1.93	12.21±1.57②	8.77±3.45①	0.72±0.07②
M	11	29.12±5.63①②	22.56±3.64	11.91±1.28②	10.11±3.72①	0.64±0.13②
H	12	30.97±3.73①②	22.33±2.34	10.57±1.48②	14.01±4.85②	0.62±0.17②

3.4 Comparison of serum biochemical indicator levels among different groups of mice The levels of blood sugar, TC, TG, ALT, BUN, Cr, and UA in the model group, as well as the levels of TC, TG, and BUN in the low and medium dose groups of honeysuckle chlorogenic acid, were significantly higher than those in the normal group (all $P<0.05$). The level of HDL-C in the model group and the level of UA in the medium and high dose groups of honeysuckle chlorogenic acid were significantly lower than those in the normal group (all $P<0.05$). The levels of blood glucose, ALT, BUN, UA among the groups of honeysuckle chlorogenic acid, the levels of TC and Cr in the middle and high dose groups of honeysuckle chlorogenic acid, as well as the level of TG in the high dose group of honeysuckle chlorogenic acid were significantly lower than those in the model group (all $P<0.05$). The levels of HDL-C in the middle and high dose groups of honeysuckle chlorogenic acid and the level of total protein in the high dose group of honeysuckle chlorogenic acid were significantly higher than those in the model group (all $P<0.05$). The results were shown as Table 3.

Under physiological conditions, the β-amyloid precursor protein (APP) secreted by neuronal cell bodies in the brain is cleaved by α-secretase, β-secretase, and γ-secretase. α-secretase hydrolyzes it within the Aβ domain of APP, producing sAPPα, which has a protective effect on neurons. The synergistic cleavage of β-secretase and γ-secretase produces Aβ1-40 and Aβ1-42, which are the main components of senile plaques (SP). According to the β-amyloid cascade theory, the formation and transformation of Aβ are at the center of the molecular mechanism of AD pathogenesis. Aβ is a common pathway that induces AD for various reasons and is a key factor in AD formation and transformation[5-6]. Currently, inhibiting β-secretase or reducing Aβ production is the main research direction for preventing and treating AD. LXA4 is an endogenous anti-inflammatory molecule with activity in inhibiting the progression of AD and improving it[7-8]. The content of AchE in the brain is closely related to brain tissue damage. An increase in its content indicates that brain tissue is damaged and repaired through nerve regeneration. However, a large amount of AchE can lead to delayed movement and may also be one of the reasons for learning and memory impairment[9].

Table 3 Comparison of serum biochemical indicators between normal and AD groups of mice ($\bar{x} \pm s$)

Group	Number	Blood sugar mmol/L	TC//mmol /L	TG//mmol/L	HDL-C//mmol/L	Total protein//g/L
Normal	13	3.45 ±0.92	1.42 ±0.23	0.75 ±0.23	14.55 ±4.10	42.63 ±24.53
Model	11	4.78 ±1.16①	2.56 ±0.66①	1.35 ±0.44①	7.96 ±5.33①	32.15 ±13.21
L	12	3.81 ±1.25②	2.28 ±0.35①	1.30 ±0.47①	8.77 ±3.45	38.45 ±21.14
M	11	3.58 ±1.09②	2.07 ±0.34①②	1.13 ±0.19①	10.11 ±3.72②	45.78 ±32.65
H	12	3.76 ±1.40②	1.79 ±0.49②	0.87 ±0.30②	14.01 ±4.85②	51.33 ±23.75②

Group	Number	ALT//IU/L	BUN//mmol/L	Cr//μmol/L	UA//μmol/L
Normal	13	20.93 ±6.27	4.88 ±1.04	160.45 ±63.48	763.86 ±155.01
Model	11	47.65 ±28.42①	9.66 ±1.64①	300.31 ±207.10①	1 062.84 ±280.44①
L	12	28.36 ±10.74②	7.97 ±0.99①②	156.33 ±36.37	836.92 ±178.07②
M	11	25.25 ±8.43②	6.15 ±1.64①②	132.70 ±15.44②	579.78 ±108.58①②
H	12	18.43 ±10.43②	5.13 ±1.22②	97.83 ±55.34②	532.09 ±147.15①②

The preliminary research of the research group found that honeysuckle can alleviate the pathological changes in brain tissue of AD model mice[10]. It can prevent excessive phosphorylation of tau protein and abnormal increase in total tau protein content by reducing Aβ1-42[2]. It has the effect of intervening in aluminum exposure induced AD and has a certain promoting effect on cardiac aluminum[11]. Its promoting effect on heavy metal excretion may be related to the chelation of organic acids in traditional Chinese medicine plants and heavy metal ions, which accelerates their excretion[12]. Chlorogenic acid is the main active ingredient in honeysuckle, which is a phenylpropanoid substance produced by plant cells through the oxalic acid pathway during aerobic respiration. Its content is an important indicator for measuring the quality of honeysuckle. In recent years, as an internationally recognized plant gold, chlorogenic acid has attracted widespread attention due to its strong biological activities, such as anti-inflammatory, antitumor, antibacterial, antioxidant, hypoglycemic, and antagonistic effects on aluminum neurotoxicity[13]. Honeysuckle chlorogenic acid is a phenolic antioxidant[14], which can inhibit Aβ-induced hippocampal neuron apoptosis, mitochondrial damage, and endoplasmic reticulum stress by enhancing antioxidant capacity, and protect neural function[15]. Cognitive function in AD mice can also be improved by enhancing the body's antioxidant capacity and reducing the expression of hippocampal APP, Aβ1-42, and phosphorylated tau protein[16]. Zhang Xinyu[17] found that chlorogenic acid can alleviate aluminum induced cell toxicity through chelation and antioxidant effects, and can exert neuroprotective effects by regulating the Akt/GSK-3β signaling pathway to reduce the production of Aβ1-42 induced by aluminum exposure.

Studies have shown that subchronic exposure to aluminum can induce an increase in expression in the cerebral cortex of mice, increase the expression of Aβ, or cause neurotoxicity through indirect channel, thereby leading to neuronal degeneration and death, and resulting in irreversible learning, memory, and cognitive dysfunction[18]. Maltol is a good ligand for aluminum and has a high affinity for aluminum. Therefore, in this experiment, maltol aluminum was used as a toxic agent to establish an AD animal model. The model established by this method is an ideal aluminum poisoning model. The results of this experiment showed that the memory ability and LXA4 content in the brain tissue of the model group mice were significantly lower than those of the normal group. The levels of β-secretase, γ-secretase, and AchE in the brain tissue were significantly higher than those in the normal group. Abnormal indicators of blood sugar, blood lipids, uric acid, and liver and kidney function were observed. Compared with the model group, the memory ability of each group of honeysuckle chlorogenic acid and the LXA4 content in the brain tissue of the high-dose honeysuckle chlorogenic acid group were significantly increased, while the contents of β-secretase, γ-secretase, and AchE in the brain tissue were significantly reduced. Moreover, the contents of β-secretase and γ-secretase could be restored to the same level as the normal group, and blood sugar, blood lipids, uric acid, and liver and kidney function indicators were significantly improved. It is speculated that the mechanism by which honeysuckle chlorogenic acid improves the learning and memory abilities of AD mice induced by aluminum toxicity may be achieved by regulating related secretory enzymes, LXA4, and various biochemical indicators.

Conflict of interest: all authors declare that there is no conflict of interest.

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has a good curative effect on ischemic stroke[15].

To sum up, this study found that Qingre Huayu Decoction may play a role in reducing cerebral ischemic injury by regulating apoptosis and autophagy, which provides a good experimental basis for the theory of "toxic damage to brain collaterals" to prevent and treat ischemic stroke.

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