

Original Article

Curcumin improves learning and memory ability via inhibiting activated microglia-mediated inflammation in mouse models of Alzheimer's disease

Zhiqiang Liu*, Yan Jiang*, Yanan Wang, Hongmei Gao, Zuoyi Chen, Liqun Fang

Department of Neurology, The Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, China. *Co-first authors.

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Abstract: To investigate the effects of curcumin on mouse learning and memory abilities in the model of Alzheimer's disease (AD), AD mouse models were established by intracranial injection of A β 1-42 and 80 male mice were randomly divided into four groups: sham group, A β 1-42 group, vehicle (0.9% saline) therapy group, and curcumin therapy group. A β 1-42 group was mice with Alzheimer's disease (AD), and vehicle (0.9% saline) therapy group and the curcumin therapy group were injected with saline and curcumin for 3 days in AD models, respectively. From 14 days, the spatial learning and memory abilities were tested by Morris water maze test. Amyloid protein (A β), GFAP, Iba-1 protein levels were measured by immunostaining and western blot assay, and the hippocampal inflammatory cytokines IL-1 β , IL-6 and TNF- α were evaluated by ELISA. A β 1-42 caused longer escape latency and worse memory compared with the sham group. Curcumin group showed significant decrease in escape latency and more passing times, and dramatically reduced A β production in comparison with A β 1-42 group and vehicle therapy group in Morris water maze. The expression of GFAP and Iba-1 in the hippocampus, as well as IL-1 β , IL-6 and TNF- α levels in the curcumin group significantly declined than those in the A β 1-42 group. Study highlights the potential of curcumin for treatment of Alzheimer's disease, which could improve learning and memory function through down-regulating inflammatory response induced by activated microglia in the presence of A β 1-42.

Keywords: Curcumin, Alzheimer's disease, inflammation, learning, memory, microglia, mice

Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder and affects over 20 million people worldwide, mainly people 65-85 year old characterized by decline in cognitive functions [1]. The pathogenesis of AD is hallmarked by features such as accumulation of amyloid beta peptide (A β), neurofibrillary tangles (NFTs) and microtubule-associated tau protein (MAPs) [2-4]. Excessive microglial cells activation have been found involved in the development of AD, including disturbance in homeostasis, neurodegeneration, neuronal cell death, synaptic loss and dysfunction, and neuroinflammation [5-7]. Activated microglial cells and accumulation A β could induce a number of neurotoxic factors, including pro-inflammatory cytokines interleukin-1 β , IL-6, tumor necrosis factor α (TNF- α),

transforming growth factor- β and nitric oxide (NO) [8, 9]. Fortunately, up to now, effective preventive and therapeutic strategy to regulate the microglia activation has been found.

Curcumin, a biologically active component of turmeric (*Curcuma longa*), is used as a flavoring spice and herbal medicine for the treatment of inflammatory conditions, wounds, AIDS and other diseases [10-12]. Recent studies have demonstrated that curcumin effectively suppressed the release of IL-1, IL-6 and TNF- α , as well as decreased the astrocytic marker GFAP and the amount of insoluble amyloid in various neuroinflammatory and neurodegenerative diseases [13-16]. Thus, curcumin is a promising agent for the treatment of Alzheimer's disease.

Therefore, present study aims to investigate the effects of curcumin on A β 1-42 induced Alzh-

Alzheimer's disease in mice, and was set forth to support the notion that curcumin might be developed as a natural anti-inflammatory agent in neurological diseases.

Material and methods

Animals

Ninety 120-day-old male Kunming mice were purchased from the Changsheng Bio technology Co Ltd (Liaoning, China; certificate number SYXK-2013-0007). Animals were maintained under controlled conditions (room temperature $25\pm 5^{\circ}\text{C}$; relative humidity 50-60%; 12 h light/dark cycle) and had free access to food and water.

Drug administration and Alzheimer model

The mice were randomly divided into four groups: each group containing 20 mice: control group (sham group) and A β 1-42 treated group (Alzheimer model group, AD group), AD/curcumin treated group (curcumin group), AD/0.9% NaCl group (vehicle group).

A β 1-42 (Dingguochangsheng Biotechnology Co., Ltd, Beijing, China) dissolved in 0.9% NaCl solution (0.25 mg/ml) for the AD group and AD/curcumin treated group, and stored at 4°C until use. Mice were anesthetized by intraperitoneal injection of 10% chloral hydrate anesthesia (50 mg/kg of body weight). The depth of anesthesia was confirmed by monitoring the lack of whisker movements and pinch withdrawal reflex prior to placement in the stereotaxic frame. Sham operated animals received only craniotomy.

A volume of 3 μl of A β 1-42 solution was stereotactically injected with a 10 μl Hamilton syringe into the right frontal lobe of the mice (AP: -0.5 mm and ML: -1.0 mm to bregma, depth 2.5-3.0 mm from dura) with a flow rate of approximate 10 minutes. Leave syringe after injection for another 10 min and move out slowly. The burr hole sealed with bone wax.

Curcumin (Sigma) dissolved in 0.9% NaCl solution administered intraperitoneally (10 mg/kg of body weight) for the AD/curcumin treated group 10 minutes after surgery for continuous three days [5, 6]. The AD/0.9% NaCl group was treated with the same volume of 0.9% NaCl solution for three days.

Memory test

The Morris water maze (MWM) was used to detect the learning and memory behavior in mice on day 14 after surgery. The water maze was a circular 100 cm, 50 cm deep, pool filled with 30 cm of water ($24\pm 1^{\circ}\text{C}$) and made opaque by the addition of nontoxic white paint. The pool was divided into four equal quadrants labeled N (north), S (south), E (east), W (west) and a platform (9 cm diameter circle, 29 cm deep) placed in the center of ES quadrants at 30 cm from the pool wall. The mice were trained to undergo a series of place navigation task of swimming to the visible platform for 4 days. The mice was placed randomly in 1 of 4 assigned quadrants and allowed to swim freely. The mice were given 60 seconds training trials for each quadrant per day (2 blocks/day). In the morning of day 5, the time (escape latency) of each quadrant the mouse needed to find the platform was recorded. In the afternoon of day 5, spatial probe training was completed where the platform was removed from the pool. The mice were allowed to search freely from the S quadrant for 90 seconds and the passing times of the mice passing through the hidden platform were recorded.

Preparation of the brain

After memory test, mice were sacrificed with an overdose of chloral. Some removed brain were immersion fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). After rinsing in PBS, brains were stored in 30% sucrose solution for at 4°C . Then coronal paraffin section was cut at 20 μm thick and stored at -20°C until used. Some hippocampi dissected from the brain for Western blot were stored at -80°C . Some hippocampi for ELISA were homogenized with 0.01 M PBS (w/v: 1:8) and stored at -80°C .

Immunohistochemistry

Briefly, brain sections were washed in 20 mM PBS, inhibited the endogenous peroxidase activity with 3% H_2O_2 for 10 min, hydrogen peroxidase for 10 minutes at room temperature, and blocked with 10% normal goat serum for 10 minutes. Slides were incubated with rabbit polyclonal anti-Iba-1 antibody (Proteintech US, 1:200) at 4°C overnight in a humidified chamber. The slides were rinsed in PBS three times and then incubated with a biotinylated goat

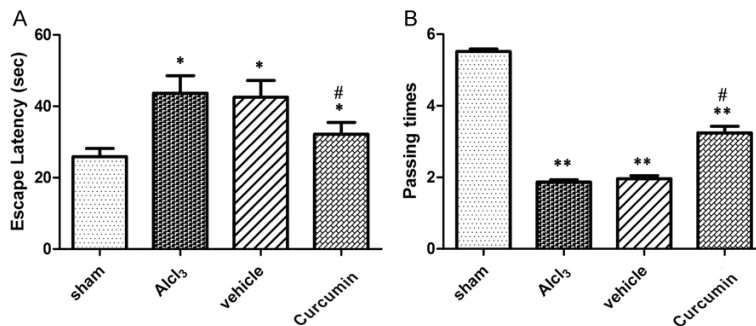


Figure 1. Aβ1-42-induced spatial memory impairment was attenuated in AD mice treated with curcumin. Morris water maze was carried out to analyze (A) the latency to reach platform (escape latency) and (B) passing times through the invisible platform. Data are presented as mean ± s.d. Statistical analysis was performed by one-way ANOVA followed by a Bonferroni test. * $P < 0.05$, ** $P < 0.01$ versus sham group. # $P < 0.05$ AD group versus AD/curcumin treated group (curcumin group).

anti-rabbit IgG secondary antibody followed by SP Kit (MAIXIN-BIO, China) manufacturer's protocol. Antigen-antibody complexes were visualized with 3,3'-diaminobenzidine (DAB) (Zhong-Shan Goldenbridge Bio, Beijing, China). Sections were mounted, cleared and coverslipped. Positive staining cells were observed under light microscope.

The sections for immunofluorescence were incubated in PBS-0.3% Triton X-100 for 10 minutes, blocked with 1% bovine serum albumin (BSA) for 30 minutes. Slides were incubated with polyclonal chicken anti-GFAP antibody (Millipore US, 1:500) at 4°C overnight. The slides were rinsed in PBS three times and then incubated with cy3 secondary antibody (1:100, Invitrogen) for 1 hour. Images were acquired on fluorescence microscope (Nikon, ECLIPSE 80i, Japan).

Immunoblotting

Western blot analysis of fresh hippocampi were lysed with RIPA buffer containing PMSF and phosphatase inhibitors (Beyotime Biotechnology, Beijing, China) and homogenized by ultrasound. After 30 min at 4°C, samples were centrifuged at 12000 rpm for 30 min at 4°C. Supernatants were isolated and protein concentration measured using a bicinchoninic acid protein kit (Beyotime Biotechnology, Beijing, China). Samples were mixed with loading buffer and boiled at 100°C for 5 min. Equal amounts of protein lysates (30 µg) were loaded and separated by 10% SDS-NuPage gel (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF)

membranes (Millipore). Membranes were blocked in 5% BSA for 1 hour, then hybridized with rabbit polyclonal antibodies against Aβ (Sigma, 1:500), Iba-1, GFAP and GAPDH (Proteintech, 1:5000) in 5% BSA-TBST overnight at 4°C, followed by HRP-conjugated secondary antibodies incubated at room temperature for 90 minutes. Detection was performed with ECL plus kit (GE-healthcare, Solingen, Germany). The bands intensity of western blot were analyzed with Image-J 5.0 software.

Protein extraction for IL-1β, IL-6 and TNF-α for ELISA

Briefly, hippocampi were homogenized by ultrasound and centrifuged at 2000 rpm for 10 min at 4°C. The levels of IL-1β, IL-6 and TNF-α in the supernatants were quantified by sandwich ELISA (R&D systems) according to the manufacturer's instruction.

Statistical analysis

All data were reported as means with standard error (SEM). Statistical analysis (differences analysis) between groups was compared with one-way ANOVA with a Bonferroni test by using SPSS version 18.0. Data visualization was performed using GraphPad Prism 5.0 Software. A P value of less than 0.05 was considered statistically significant.

Results

Aβ1-42-induced spatial memory impairment was attenuated in AD mice with curcumin treatment

To analyze the effects of curcumin on the spatial memory of AD mice, escape latency and passing times were monitored in AD mice and AD/curcumin treated mice using morris water maze. Aβ1-42 treated mice showed significant increase in escape latency as compared to the sham group, suggesting AD mice take much longer time to reach the visible platform ($P < 0.05$) (Figure 1A). In AD/curcumin treated group, the escape latency to find the platform was shortened significantly ($P < 0.05$ v.s. AD group). Moreover, AD/curcumin treated group signifi-

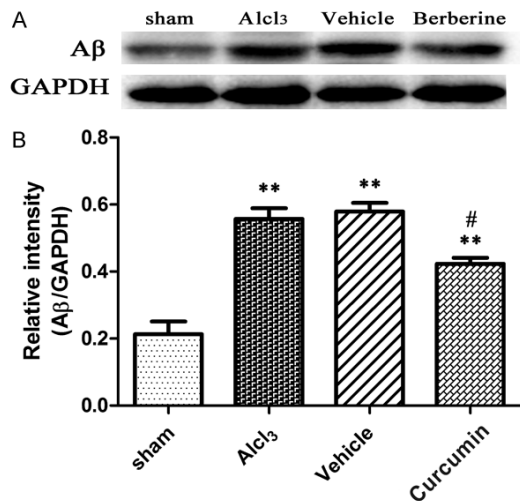


Figure 2. Curcumin decreased A β protein accumulation in the hippocampus. A and B. Immunoblot analysis of A β protein in the hippocampus. Data are presented as mean \pm s.d. Statistical analysis was performed by one-way ANOVA followed by a Bonferroni test. ** $P < 0.01$ versus sham group. # $P < 0.05$ AD group versus curcumin group.

cantly increased passing times to the invisible platform compared to the AD group ($P < 0.05$) (Figure 1B).

Curcumin decreased A β protein accumulation in the hippocampus

It is clear that A β protein level in the hippocampus was higher in the AD group than that in the sham group ($P < 0.01$). After treating with curcumin for 3 days, a significant decrease could be seen in the hippocampus of AD/curcumin treated group ($P < 0.05$ v.s. AD group) (Figure 2).

Curcumin reduced the expression of Iba-1 and GFAP in the hippocampus of AD mice

Immunohistochemistry staining shows that increased Iba-1 and GFAP expression levels were detected in the brain of the AD group ($P < 0.01$, respectively v.s. sham group), indicating activation of microglia and astrocytes are involved in the Alzheimer brain (Figure 3A-D). On the other hand, curcumin showed an effect reduction of Iba-1 and GFAP expression in the hippocampus region ($P < 0.05$, respectively v.s. AD group). To validate this finding, the Iba-1 and GFAP protein levels in the hippocampi were analyzed by western blot. Noteworthy, AICl3 induced higher levels of Iba-1 and GFAP. In comparison, curcumin treated AD mice showed 20% reduction

in Iba-1 and GFAP levels respectively ($P < 0.05$, respectively) (Figure 3E).

Curcumin treatment down-regulated the AICl3-induced pro-inflammatory cytokines

Further, ELISA was performed in order to investigate whether curcumin regulates pro-inflammatory cytokines expression. AICl3 significantly elevated IL-1 β , IL-6 and TNF- α levels in the hippocampus ($P < 0.01$, respectively v.s. sham group). Conversely, curcumin treatment reversed the effect of AICl3-induced IL-1 β , IL-6 and TNF- α levels in hippocampus ($P < 0.05$, respectively v.s. AD group) (Figure 4).

Discussion

In present study, we investigated the effects of curcumin on AICl3-induced Alzheimer's disease. There are evidences confirming that aluminium exposure is a potential risk factor for elderly cognitive impairment and progression of AD [17]. Also, aluminium caused cognitive dysfunction in animals [18]. Similar to these our results showed AICl3 exposure resulted in a significant decrease in spatial memory as indicated by elevated escape latency and reduced times of passing through the invisible platform. However, the treatment with curcumin reversed the spatial memory deficit induced by AICl3, suggesting the beneficial effects of curcumin in correcting memory deficit associated with aluminium exposure.

Our study also has implications on pathogenic mechanisms in AD. AICl3 exposure stimulated microglia and astrocyte, as well as the increase of A β protein in mice brain. Activation of these immune cells resulted in the release of inflammatory mediators (IL-1 β , IL-6 and TNF- α). Previous studies have been demonstrated that the activated microglial cells produce A β protein, secrete the pro-inflammatory cytokines and trigger free radicals production [8, 19]. Further, the accumulation of A β plaques could stimulate the microglial cells, astrocytes and monocytes to produce neurotoxic compounds such as glutamate [20, 21]. Thus, prevention of AD progression through modulation of microglia inhibition is a potential at least in experimental and clinical settings.

During recent years several herbal compounds or its active constituent, such as berberine, Huanglian-Jie-Du-Tang-M (HLJDT-M, without Radix scutellariae) and coptisine, have been be-

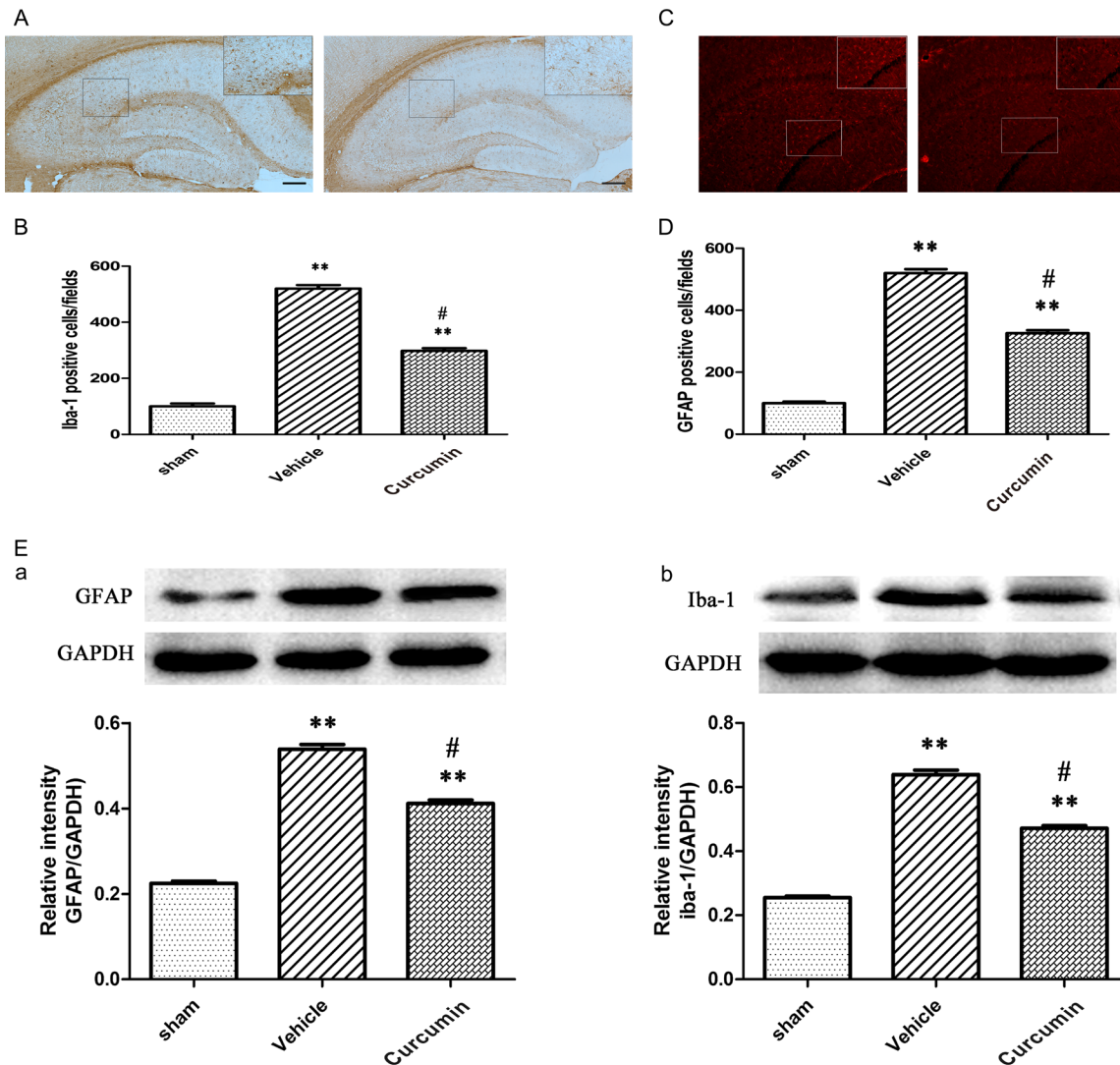


Figure 3. Curcumin reduced the expression of Iba-1 and GFAP in the hippocampus of AD mice. A and C. Immunostaining of Iba-1 (brown) and GFAP (red) protein in the mice brain. Upper, higher magnification of area within the hippocampus. Scale bar represents μm . B and D. Quantification of Iba-1 and GFAP protein expression was subsequently performed. E. Immunoblot analysis of Iba-1 and GFAP protein in the hippocampus. Data are presented as mean \pm s.d. Statistical analysis was performed by one-way ANOVA followed by a Bonferroni test. ** $P < 0.01$ versus sham group. # $P < 0.05$ AD group versus curcumin group.

coming center of interest. They are observed to suppress proinflammatory responses in microglia and reduce A β plaques generation *in vitro* and *in vivo* [22-25]. Curcumin is a natural anti-inflammatory and antioxidant compound. There are evidence demonstrated that curcumin could protect neurons, attenuate synaptic dysfunction and promote hippocampal neurogenesis [26-28]. In line with observation in other studies curcumin administration enhanced antioxidant and anti-inflammatory capacities [12]. Its beneficial effects might be related to

its inhibition to the inflammatory mediators produced by activated microglia.

Here we noted that curcumin significantly suppress the microglia and astrocyte activation. Our data also provided evidence for the anti-inflammatory properties of curcumin in inhibiting both A β aggregation and the transcription of proinflammatory cytokine genes in the AD hippocampus. These results were consistent with a recent study that curcumin suppressed production of inflammatory mediators in A β -

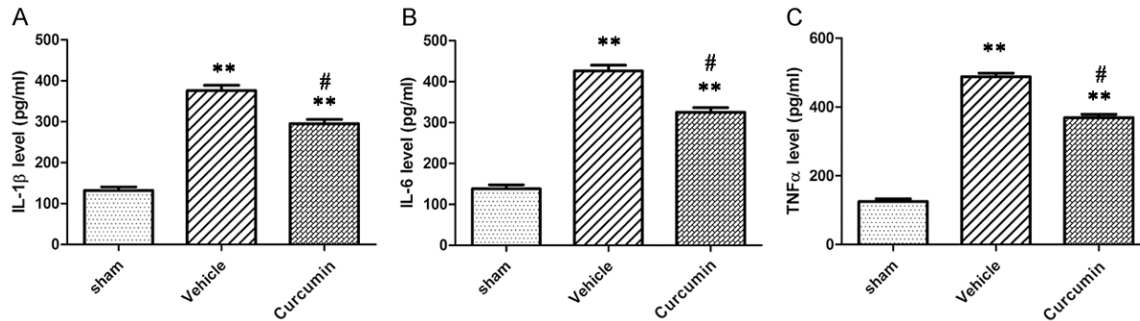


Figure 4. Curcumin treatment down-regulated Aβ1-42-induced pro-inflammatory cytokines. A-C. IL-1β, IL-6 and TNF α levels in the hippocampus were evaluated with ELISA. Data are presented as mean ± s.d. Statistical analysis was performed by one-way ANOVA followed by a Bonferroni test. ** $P < 0.01$ versus sham group. # $P < 0.05$ AD group versus curcumin group.

induced primary mouse microglia *in vitro* [28]. Moreover, it is found that MAPK, ERK1/2 and p38 pathways might be involved in the Aβ-induced activated microglial inflammatory responses, which may attribute to the up-regulated expression of inflammatory mediators [29]. However, treatment with curcumin could suppress the activation of these pathways, playing a protective role in the inflammatory and oxidative processes in Alzheimer's brain [29-31].

Therefore, in this study we investigated whether curcumin treatment can successfully fight activated microglia and astrocyte and whether curcumin can efficiently reverse inflammatory processes in Alzheimer's disease. Our results suggest that application of curcumin does not only reduce excessive neuroimmune cells growth and properties but also improve the learning and memory functional activities in AD mice.

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Disclosure of conflict of interest

None.

Address correspondence to: Liqun Fang, Department of Neurology, The Fourth Affiliated Hospital of Harbin Medical University, No. 37, Yiyuan Street, Nangang District, Harbin, China. E-mail: fsahdy@sina.com

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