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Matrine ameliorates cognitive deficits via inhibition of microglia mediated neuroinflammation in an Alzheimer's disease mouse model

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Amyloid β (Aβ) induced microglial activation and attendant neuroinflammation play pivotal roles in Alzheimer's disease (AD) pathogenesis. Matrine is a natural anti-inflammation compound from the Chinese herbal medicine *Sophora flavescens* Ait. (Kushen). This study aimed to investigate the effects of matrine on memory deficit and neuroinflammation in an oligomeric Aβ (oAβ)-induced AD mice model. Whether microglial activation and NADPH oxidase were involved in these effects were further studied. Different doses of matrine (10, 20, or 40 mg/kg) were intragastrically administered once a day after intracerebroventricular oAβ injection (2.5 μg/μl, 4 μl). 15 days after the oAβ injection, behavioral experiments including novel object recognition (NOR) test and Morris water maze (MWM) test were performed. 21 days after the oAβ injection, concentration of ROS, TNF-α, IL-1β and IL-6 as well as expression of NADPH oxidase subunits gp91phox and p47phox in mice hippocampal tissues were assessed, and microglial activation were evaluated by Iba-1 immunohistochemical staining. Results of NOR test and MWM test revealed that oAβ injection could remarkably impair learning and memory function in AD mice, and matrine administration could significantly ameliorate the impairment. ROS, TNF-α, IL-1β and IL-6 levels increased after oAβ injection, while matrine could significantly reduce the concentrations of these inflammatory factors. OAβ induced protein expression of NADPH oxidase subunits gp91phox and p47phox were also significantly reduced by matrine. Iba-1 immunohistochemistry results showed less activated microglia in matrine-treated mice brain. These results indicate that matrine could ameliorate learning and memory impairment and neuroinflammation induced by oAβ injection. These effects were found to be mediated through inhibition of microglial activation and NADPH oxidase expression in hippocampal tissue. The results suggest that matrine may be a valuable natural compound with therapeutic potential against AD.

1. Introduction

Alzheimer's disease (AD) is the most common age-associated neurodegenerative disorder causing progressive dementia. Neuroinflammation mediated by microglia plays a key role in AD pathogenesis cascade (Regen et al. 2017). Microglia are resident immune cells in the brain which play an important role in host defense and tissue repair in the central nervous system (CNS) (Mosher et al. 2014). In response to brain injury or immunological stimuli, such as amyloid beta (Aβ) or lipopolysaccharide (LPS), microglia are activated, thus producing varieties of pro-inflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, nitric oxide (NO), and reactive oxygen species (ROS) (Wang et al. 2015; Zhang et al. 2015). Accumulation of these mediators contributes to neuronal damage and aggravates AD progression. Therefore, the production of inflammatory factors activated by microglia should be inhibited to prevent neuro-inflammation and neurodegeneration in AD. In the last 20 years, both laboratory and epidemiologic studies have proved that anti-inflammatory medications including aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) prevent or defer AD process (Deardorff et al. 2016; Woodling et al. 2018; O'Bryant et al. 2018). Some small molecular compounds derived from natural products, such as tripchlorolide have also shown the ability to protect neurons through inhibiting Aβ-induced microglial activation (Pan et al. 2009; Wang et al. 2016). These observations suggest that drugs targeting inflammatory processes may serve as potential therapeutic approaches in AD, and anti-inflammatory natural compounds may have a great potential for the development of anti-AD drugs.

Matrine is a quinolizidine alkaloid, which is one of the major active components of the Chinese herbal medicine *Sophora flavescens* Ait. (Kushen). It has been shown to possess a variety of pharmacological effects including anti-inflammation, immune regulation, antiviral and anti-tumor properties (Zhang et al. 2011; Yang et al. 2015; Yang et al. 2012), leading to wide clinical use for the treatment of several diseases such as enteritis, viral hepatitis, liver fibrosis and atopic dermatitis in China. It was also reported that matrine could effectively protect neuro-axons from CNS inflammation-induced damage (Kan et al. 2015). Regarding the

key role of neuroinflammation in AD pathogenesis, we hypothesized that matrine might protect brain damage in AD, depending on anti-inflammatory effects. Thus, we demonstrated the *in vivo* effects of matrine on memory deficit and neuroinflammation in oAβ-induced AD mice model. To elucidate the mechanism underlying the anti-inflammatory action, the effect of matrine on microglial activation and NADPH oxidase subunits expression *in vivo* were further assessed.

2. Investigations and results

2.1. Matrine ameliorates oAβ induced learning and memory impairment

NOR test were performed to evaluate the effect of matrine in an oAβ-injected mouse model. The oAβ-injected group showed significantly reduced preference index, while matrine and donepezil treatment groups exhibited a remarkable recovery in novel object recognition ability (Fig. 1A). To confirm the protective effect of matrine on learning and memory function, a Morris water maze (MWM) test was performed. In the place navigation test, mice injected with oAβ displayed significantly longer escape latency

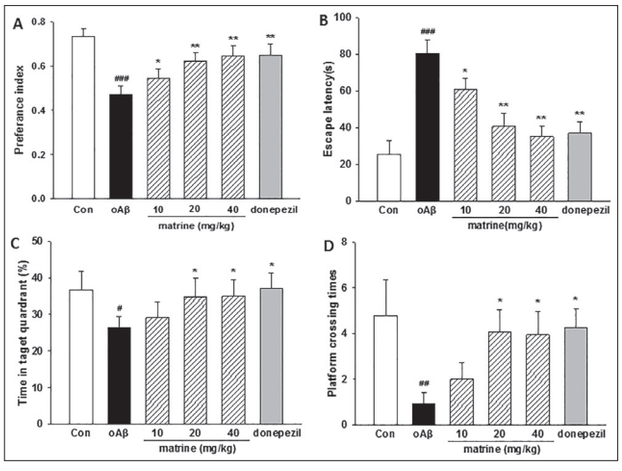

Fig. 1: Effects of matrine on learning and memory function in oAβ-induced AD mice. OAβ-injected AD mice were orally treated with saline, matrine (40, 20 and 10 mg/kg) or donepezil (5 mg/kg). (A) Preference index (PI, %) in NOR test. (B) Escape latency during spatial acquisition training. (C) The time spent in target quadrant and (D) platform crossing times during spatial probe test. Each bar represents the mean±S.E.M. of data (n =10). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with control group. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with oAβ group.

compared to those in the control group, which can be reversed by matrine and donepezil (Fig. 1B). In the spatial probe test, both the time spent in the target quadrant and platform-crossing times decreased in oAβ-injected mice, which can be reversed by matrine and donepezil treatment (Fig. 1C and 1D). For the first time, we found that administration of matrine can ameliorate Aβ induced learning and memory impairment in AD mouse model.

2.2. Matrine inhibits oAβ induced pro-inflammatory cytokines release in vivo

The effects of matrine on the production of oAβ-induced pro-inflammatory cytokines in mice hippocampus were investigated. We found that oAβ injection caused significant increase in the release of ROS, TNF-α, IL-1β and IL-6 compared with the control group mice. While the increased levels of these pro-inflammatory cytokines in hippocampus were dramatically diminished after matrine treatment (Fig. 2). The results indicate that oAβ peptide induced neuroinflammation could be effectively inhibited by matrine.

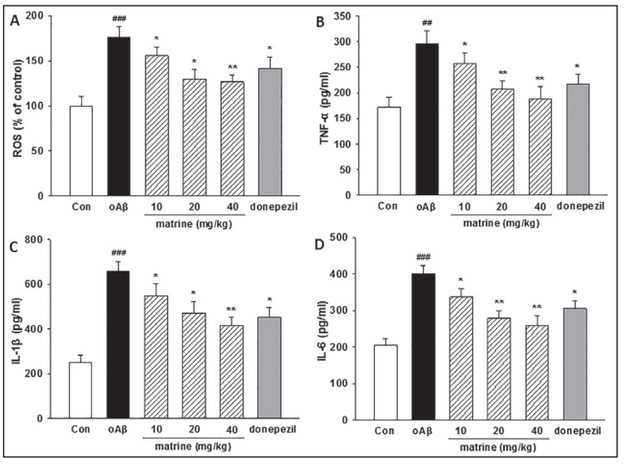

Fig. 2: Effects of matrine on oAβ-induced pro-inflammatory mediators release in AD mice. OAβ-injected AD mice were orally treated with saline, matrine (40, 20 and 10 mg/kg) or donepezil (5 mg/kg) for 21 days. Then the mouse brain hippocampal tissues were collected for ROS (A), TNF-α (B), IL-1β (C) or IL-6 (D) concentration measurement. Each bar represents the mean±S.E.M. of data from three independent experiments (n = 3). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with oAβ group.

2.3. Matrine inhibits oAβ-induced microglial activation in vivo

To examine the effects of matrine on hippocampal microglial activation and neuronal loss in oAβ-injected mice, immunohistochemical experiments were performed. It is found that the Iba-1 positive areas were significantly increased in the oAβ-injected mice compared with those in the sham group. With the treatment of matrine, the Iba-1 positive areas were remarkably decreased (Fig. 3). The results indicate that matrine could effectively inhibit microglial activation *in vivo*.

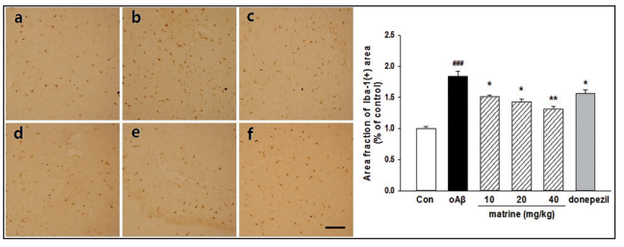

Fig. 3: Effects of matrine on oAβ induced microglial activation in vivo. OAβ-injected AD mice model were orally treated with saline, matrine (40, 20 and 10 mg/kg) or donepezil (5 mg/kg) for 21 days. Then the mouse brain hippocampal tissues were collected and activation of microglia was visualized by Iba-1 staining (A). Quantification analysis of the Iba-1-stained cells (B) was conducted by measuring the fractions of Iba-1-immunoreactive areas in the DG. Values are expressed as mean±S.E.M.. $^{\#\#\#}P < 0.001$ compared with control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with oAβ group.

2.4. Matrine suppresses the protein expression of NADPH oxidase subunits gp91phox and p47phox

NADPH oxidase plays a key role in microglia mediated neuroinflammation. Activation of NADPH oxidase required cytosolic subunits (p47phox, p67phox, and p40phox) and catalytic subunits gp91phox and p22phox (Ma et al. 2017). Western blot analysis showed that the presence of oAβ increased the protein expression of gp91phox and p47phox in AD mice brain, while this increase was prevented by matrine treatment (Fig. 4). Thus, matrine may inhibit oAβ-induced microglial activation and neuroinflammation by inhibiting the expression of NADPH oxidase subunits gp91phox and p47phox.

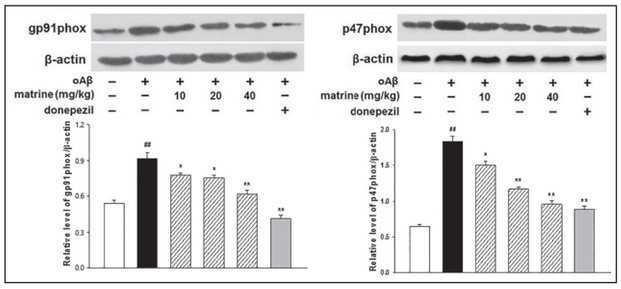

Fig. 4: Effects of matrine on oAβ induced protein expression of NADPH oxidase subunits gp91phox and p47phox in microglial cells. OAβ-injected AD mice were orally treated with saline, matrine (40, 20 and 10 mg/kg) or donepezil (5 mg/kg) for 21 days. Then the mouse brain hippocampal tissues were collected and gp91phox and p47phox protein expressions were evaluated by western blotting assay. Expression levels of the β-actin were used for standardization, and ImageJ software (version 1.44) was used for band pattern analysis. Values are expressed as mean±S.E.M.. $^{\#\#}P < 0.01$ compared with control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with oAβ group.

3. Discussion

In this study, we demonstrated that matrine could ameliorate learning and memory impairment in oAβ-induced AD mice model. The downregulation of NADPH oxidase subunits gp91phox and p47phox expression by matrine decreased the pro-inflammatory factors released by microglial cells such as ROS, TNF-α, IL-1β and IL-6. These findings suggest that matrine ameliorated oAβ-induced cognitive deficits and neuroinflammation by inhibiting microglia activation and protein expression of NADPH oxidase subunits. Similar to matrine, donepezil also showed significant effects on inhibition of neuroinflammation and down-regulation of NADPH oxidase subunits expression, which is consistent with the previous research (Kim et al. 2014; Hwang et al. 2010).

Although numerous studies have revealed that matrine is a potent inhibitor of inflammation, the effect of matrine on neuroinflammation is not well elucidated. In rats with experimental autoimmune encephalomyelitis, matrine protects neuro-axon from CNS inflammation-induced injury through inhibition of β-secretase-1 (BACE1) expression and upregulation of brain-derived neurotrophic factor (BNDF) (Kan et al. 2015). Matrine was also reported to exert direct protection of neurons by inhibition of the NF-κB signaling pathway against focal cerebral ischemia (Xu et al. 2012). Moreover, it was shown that matrine could inhibit LPS-induced microglial activation *via* suppressing the HSP60 signaling pathway (Zhang et al. 2017). Results of this study suggested that oral administration of matrine significantly ameliorates the neuroinflammation triggered by oAβ in mice hippocampus, providing more evidence for the anti-inflammatory effect of matrine in central nervous system.

Recent studies have shown that microglia are the main resources of NADPH oxidase in the brain (Vilhardt et al. 2016; Hou et al. 2017). Among various neurotoxic factors produced by activated microglia, NADPH oxidase-derived ROS play an important role in microglia-mediated neuroinflammation. ROS participate in host defense by destroying invading pathogens and inducing a variety of antioxidant enzymes in host cells (Jain et al. 2013). Recent studies have revealed that ROS also serve as secondary messengers to enhance gene expression by encoding a variety of pro-inflammatory factors (Bakunina et al. 2015). In this study, we found that matrine reduced ROS production in oAβ-injected mice brain. Although matrine was reported to reduce oxidative effects by functioning as an ROS scavenger (Pang et al. 2016), a new finding from the present study was that matrine could inhibit oAβ-induced protein expression of microglial NADPH oxidase subunits gp91phox and p47phox, leading to decreased ROS production. Although matrine affects expression of gp91phox, which is the dominant NADPH oxidase and the major superoxide-generating enzyme in microglia, the action of matrine on the phosphorylation and translocation of NADPH oxidase subunits to influence NADPH oxidase activity remains to be further investigated.

Matrine also showed potent inhibitory effects on the production of TNF-α, IL-1β and IL-6 in the oAβ-injected mice brain. These inflammatory factors are regarded as remarkable substances in microglial activation (Zhang et al. 2010; Salemme et al. 2016). Our previous study showed that stand-alone oAβ could induce the production of the three pro-inflammatory factors in microglia, and NADPH oxidase play an important role in these effects (Li et al. 2013). Accordingly, the inhibition of NADPH oxidase by matrine reduced pro-inflammatory factors released by the oAβ-activated microglial cells. Recently it is widely accepted that these increased cytokines and chemokines released by activated microglia, which cause chronic neuroinflammation, are partly responsible for neuronal damage and neurodegeneration in AD brain. Thus, the inhibitory effects of matrine on oAβ-induced pro-inflammatory factor release partly contributed to its neuro-protective and cognitive improvement effects against AD.

This study demonstrated that matrine inhibited oAβ-induced microglial activation, resulting in the inhibition of neuroinflammation and protection of cognitive function. Mechanistic study showed that the inhibitory effects of matrine on the activation of microglia were mediated by NADPH oxidase. Moreover, NADPH oxidase subunits gp91phox and p47phox played important roles in these effects. The results suggest that matrine is a valuable natural product with therapeutic potential against AD.

4. Experimental

4.1. Preparation of peptides

Aβ1-42 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). OAβ were prepared as described in our previous paper (Li et al. 2013).

4.2. Regents

Matrine were purchased from Ningxia Bauhinia Pharmaceutical Co., Ltd. (Yinchuan, China), with 98% purity (detected by high performance liquid chromatography, HPLC). Enzyme linked immunosorbent assay (ELISA) Kits for determining TNF-α, IL-1β, IL-6 and ROS were purchased from BOSTER Biological Technology Co. Ltd. (Wuhan, China). The antibodies including gp91phox, p47phox and β-actin were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

4.3. Animals and drug administration

Male C57BL/6 mice at 8 weeks old, weighing about 22-25 g (Experimental Animal Center of Ningxia Medical University, Yinchuan, China) were housed under standard conditions at 25 °C with a 12 h/12 h dark/light cycle, with food and water freely available. All experiments and animal care were conducted in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by Ethic Committee of Ningxia Medical University (NXMU2014-124). Mice were anesthetized by intra-peritoneal injection of 350 mg/kg chloral hydrate and placed in the stereotaxic apparatus. Each mouse was intracerebroventricularly (i.c.v.) injected with 2 nmol oAβ (4 μL) according to the stereotaxic atlas of mouse brain (Souza et al. 2017). The control group mice received were injected with the same volume of saline. One day after injection, the total of 60 mice were randomly divided into six groups (10 mice per group): control group (sham-operation with oral administration of saline), model group (oAβ injection with oral administration of saline), donepezil group (oAβ injection with oral administration of donepezil 5 mg/kg/d) and matrine groups (oAβ injection with oral administration of matrine 40, 20 and 10 mg/kg/d respectively). Animals were observed for 21 days while the novel object recognition (NOR) test and the Morris water maze (MWM) test were performed 15~21 days after oAβ injection. After behavioral experiments, the animals were sacrificed by cervical dislocation at day 22. Mice brain and hippocampus were collected. Iba-1 immunohistochemistry analyses were performed, and concentration of ROS, TNF-α, IL-1β and IL-6 were measured using ELISA kits. Expression of NADPH oxidase subunits gp91phox, p47phox in hippocampal tissue were assessed with western blot analysis.

4.4. Novel object recognition (NOR) test

The NOR tests were carried out as described previously (Fukumoto et al. 2014). The experiments were performed in a black open test box (50 cm × 50 cm × 30 cm). Firstly, all animals were placed in the box without objects for 3 min to habituate to the environment. After the habituation period, two identical objects were placed into the test box and mice were allowed to explore for 3 min. The time spent by the mice exploring each object was recorded (defined as the training session). After a 24 h delay, one of the original objects used in the training session was replaced by a novel object (different in shape and color, similar in size) and mice were allowed to explore in the box for 3 min. The time spent by the mice exploring the novel and the familiar objects were measured respectively (defined as the test session). The mice were regarded to be exploring when they were sniffing or touching the object. Preference index (PI, %) were calculated as the percentage of novel object exploring time during test session.

4.5. Morris water maze test

To evaluate the effects of matrine on oAβ induced spatial learning and memory dysfunction, Morris water maze (MWM) test was performed as described before (Yang et al. 2018). Briefly, the tank (1.0 m in diameter) was divided into four quadrants and a hidden transparent platform (6.5 cm in diameter) was fixed in the center of the IV quadrant 0.5 cm below the water surface. The place navigation training lasted for three consecutive days. Mice were individually released into water in each quadrant at the water-level, facing the pool wall and allowed to swim freely to find the hidden platform. Mice were placed onto the platform for 30 s if they could not find the platform within 90 s. Mice were trained three times each day. The place navigation test was performed 24 h after the last training and escape latency (the time taken to find the platform) was recorded. Spatial probe test was performed after the place navigation test, in which the platform was removed and mice were individually released into pool from the II quadrant. Each animal was allowed to swim 90 s to search the platform. Time spent in target quadrant and platform-crossing times were recorded and analyzed by the analysis-management system (WMT-100S Analysis System, Techman Software, Chengdu, China).

4.6. Determination of ROS, TNF-α, IL-1β and IL-6

The mice hippocampal tissues were homogenized in ice-cold homogenization buffer and then homogenates were centrifuged at 10,000 g for 10 min at 4 °C. The concentrations of ROS, TNF-α, IL-1β and IL-6 in the supernatant were measured using ELISA kits according to the manufacturer's protocol.

4.7. Western blotting assay

Hippocampal tissues of sacrificed mice were lysed with a triple-detergent lysis buffer to detect gp91phox and p47phox expression. The protein concentration in the supernatant fluid of the lysate was measured by the BCA protein assay (Pierce, Rockfold, IL, USA). Equal amounts (60 μg) of protein were separated electrophoretically by 12% SDS-PAGE (Ameresco, Solon, OH, USA) and then the gel was transferred to 0.45 μm polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membranes were soaked in blocking buffer (5% skimmed milk) and then incubated overnight with primary antibodies (gp91phox, 1:500 dilution; p47phox, 1:500 dilution) at 4 °C, followed by incubation with horseradish peroxidase conjugated secondary antibodies (1:4000 dilution, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) for 1 h at room temperature. Following three washes in Tris-buffered saline-Tween (TBST), immunoreactive bands were visualized using the enhanced chemiluminescence reagent (ECL, Beyotime Institute of Biotechnology, Shanghai, China). Expression levels of the β-actin were used for standardization. ImageJ software (version 1.44, National Institutes of Health, Bethesda, MD, USA) was used for band pattern analysis.

4.8. Immunohistochemistry staining

The free floating sections were rinsed in PBS and treated with 1% hydrogen peroxide for 15 min to remove endogenous peroxidase activity. Then, they were incubated separately with a rabbit anti-Iba-1 (1:800 dilution, Wako, Osaka, Japan) antibody overnight at 4 °C in the presence of normal goat serum and 0.3% triton X-100. Next, they were subjected to another incubation with biotinylated anti-rabbit (1:200 dilution, Vector, Burlingame, CA, USA) for 90 min, followed by incubation in avidin-biotin complex (1:100 dilution) for 1 h at room temperature. Peroxidase activity was visualized using DAB in 0.05 M tris-buffered saline (pH 7.6). The images were captured by an optical light microscope (BX51, Olympus, Tokyo, Japan) at 200x magnification to quantify the immunoreactivity of Iba-1 in the hippocampus. ImageJ software was used to analyze the average area fractions of Iba-1-stained regions. Selection of Iba-1 positive area was conducted by manual threshold adjustment. The fraction of immunoreactive region was calculated as the selected area divided by captured total area. The data were expressed as percentages of the value compared to the vehicle-treated control group.

4.9. Data analysis

Results were expressed as the mean±S.E.M. ANOVA and Student's t test were used for statistical analysis (SPSS 16.0; SPSS, Chicago, IL, USA). Differences with p values less than 0.05 were considered to have statistical significance.

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Conflicts of interest: None declared.

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