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Oxymatrine can attenuate pathological deficits of Alzheimer's disease mice through regulation of neuroinflammation

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A B S T R A C T

Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by progressive learning and cognitive damage. Several hypotheses such as amyloid cascade hypothesis, hyper-phosphorylated τ hypothesis, and energy metabolism hypothesis have been proposed to elucidate the disease. However, the exact mechanism of AD remains unclear and current therapeutic strategies are miserable. Cumulative evidence showed that neuroinflammation plays a significant role in the pathogenesis of the AD. Oxymatrine (OMT), a plant-derived bioactive compound, has anti-viral, anti-fibrosis, and anti-tumor effects through the involvement of several immune-related signaling pathways. Whether OMT can attenuate the pathology of AD is largely unknown. In this manuscript, we found that treatment of OMT can significantly improve cognitive and learning abilities of AD mice during various behavioral test. Treatment of OMT can significantly reduce the densities of Aβ plaques and astrocyte clusters in the neocortex and hippocampus of AD mice. Furthermore, treatment of OMT significantly reduced the concentration of pro-inflammatory cytokines including IL-6, IL-1β, TNF-α and IL-17A in AD mice. Taken together, our data indicate that OMT may serve as a potential drug for AD.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder clinically characterized by progressive decline in memory, cognitive competence, and locomotor activities (Alzheimer's Association, 2016). Aging is the most prominent pathogenic factor of AD. However, many diseases and lifestyle factors may contribute to the development of the AD, such as genetics, depression, epilepsy, brain trauma, obesity and hypertension (Ballard et al., 2011; Scheltens et al., 2016; Stern et al., 1995; Swaminathan and Jicha, 2014). The elusive mechanism of AD makes it still difficult to find an effective therapeutic strategy (Cummings et al., 2017; Waite, 2015).

The immune system is the key defender of human body and plays a crucial part in the homeostasis of the central nervous system (CNS) (Bilbo and Schwarz, 2012). Cumulative evidence indicates that neuroimmune hemostasis and neuroinflammation are involved in the onset and the development of neurodegenerative diseases (De Luca et al., 2018; Morales et al., 2014; Newcombe et al., 2018; White et al., 2017). After the initiation of brain damage or injury, the immune cells in the CNS, mainly astrocyte and microglia are recruited to the insulted area (Shastri et al., 2013). These cells then secrete a series of pro-inflammatory elements like cytokines, cytotoxic elements and reactive oxygen species (ROS), which eventually lead to the phagocytosis of debris and pathogens or other immune responses (Bradt et al., 2000; Frautschy et al., 1992). In normal conditions, the acute inflammatory response return to the baseline quickly after the clearance of debris (Fullerton and Gilroy, 2016). However, in AD conditions, acute inflammatory responses are prolonged into chronic inflammation which lead to the over loss of neurons (Streit et al., 2004). Currently, amyloid cascade hypothesis and hyper-phosphorylated τ hypothesis are the main hypothesis for AD (Du et al., 2018). $A\beta_{40}$ and $A\beta_{42}$ are produced by differential cleavage of the amyloid precursor protein (APP) by γ-secretase. Peptide $A\beta_{42}$ is more likely to form aggregates and more toxic to the central neural system than $A\beta_{40}$ (Butterfield et al., 2013; Mucke and Selkoe, 2012; Takahashi et al., 2017). The pathological inflammatory course in AD is largely facilitated by the interaction between $A\beta_{42}$ oligomers/hyper-phosphorylated τ aggregates and reactive astrocyte/microglia (Cai et al., 2017; Laurent et al., 2018; Mathur et al., 2015). Considering the central role of neuroinflammation in the pathogenesis of AD, effective therapeutic strategies may be based on anti-inflammatory treatment.

Oxymatrine (OMT) is an alkaloid component extracted from the root of *Sophora flavescens Ait* ("kushen", a traditional Chinese medicine) and has a tetracyclic quinolizine structure (Fig. 1A). Plenty of researches have shown that OMT has anti-viral, anti-apoptotic, anti-fibrosis, and anti-tumor effects (Li et al., 2017; Liu et al., 2009; Lu et al.,

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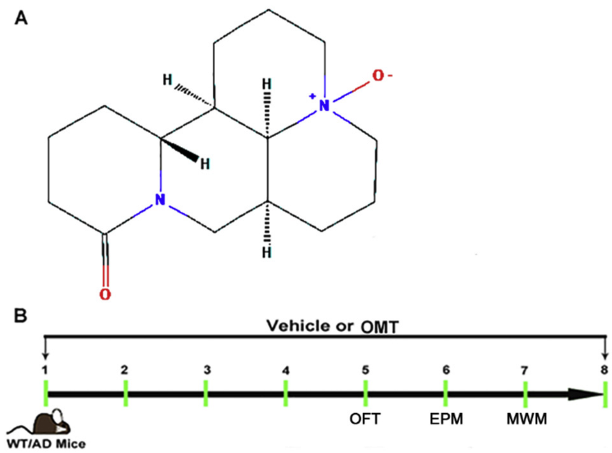

Fig. 1. Molecular structure of oxymatrine (OMT) and behavioral tests strategy. (A) Molecular structure of the oxymatrine (OMT). (B) Schematic of behavioral tests strategy for the effect of OMT on AD mice.

2003; Wu et al., 2017; Zhao et al., 2018). In addition, OMT has been used as a strong anti-inflammatory agent in clinical use for many years. Several potential signaling pathways are involved in the anti-inflammatory effect of OMT such as TLR4, JAK/STAT and NF-κB (Guzman et al., 2013; Lu et al., 2016). Previous works clearly demonstrated that the administration of OMT can significantly downregulate the level of pro-inflammatory factors like TNF-α, IL-1β, and IL-6 (Dong et al., 2013; Mao et al., 2012; Wen et al., 2014). Oxidative stress is another contributor to the development of AD. OMT can also reduce the oxidative stress damage in a dose dependent manner *via* NF-κB pathway (Cheignon et al., 2018; Yang et al., 2014). It has a long history in China to use the mixtures of “kushen” and some other Chinese traditional medical herbs to treat dementia and mental disorders. Besides, recent study has shown that OMT can attenuate neurotoxicity induced by $A\beta_{1-42}$ in primary neuronal cells and memory impairment in rats (Dong et al., 2018). Nevertheless, the protective effect of OMT to AD still lack direct evidence. In the present study, we investigated the protective effect of OMT to hAPP AD mice both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Animals and oxymatrine treatment

Eight-month-old male hAPP transgenic mice and wild type were used in this study. hAPP transgenic mice (JAX: 030670) were purchased from Jackson Laboratory and wild type C57BL6/L mice were purchased from Shanghai Laboratory Animal Centre (SLAC). All animals were raised under a 12 h light/dark cycle with food and water *ad libitum*. All animal experiments were under the guidance and supervision of Affiliated Hospital of Jining Medical University.

Oxymatrine (CC2494) were purchased from Chemcatch and dissolved in sterilized 0.9% NaCl solution into a final concentration of 50 mg/mL. Oxymatrine or vehicle (0.9% NaCl solution) were intraperitoneally injected to hAPP mice (OMT, 40 mg/kg) daily for consecutive 8 weeks before sacrificing.

2.2. Behavioral tests

After the initial oxymatrine injection, four groups of mice (WT, AD mice, AD + OMT mice and AD + Veh mice, each group containing 6 mice) were assigned to perform open/closed field test, elevated plus maze test and Morris water maze test on the fifth, sixth and seventh week respectively. Only animals with comparative weight were chosen for behavioral tests to exclude individual difference. Experimenters were blind to the group assignment and did not participate the later data analysis. All mice were adapted to the testing room for at least 30 min before experiments.

2.3. Open/closed field test

The open field apparatus is composed of a white square plastic box (45 cm × 45 cm) which is divided into 9 small arenas. The box floor is illuminated by LEDs and monitored by a camera equipped with Noldus Ethovision 8.5 software. The “closed field test” is when the box lid is closed and without illumination. Each animal was gently placed in the box and allowed to freely explore in the box for 15 min. The total distance moved and the number of small arenas crossed were recorded and analyzed.

2.3.1. Elevated plus maze test

During the elevated plus maze test (EPM), the mouse was gently placed in the center of the maze with its head towards the open arm. The spontaneous activity of the tested mouse was monitored for 5 min by a camera equipped with Video Maze and Video Tracking Interface. The percentage of open arm entry and open arm time were recorded and analyzed.

2.3.2. Morris water maze test

Morris water maze test is the classic behavioral model for memory and learning test. On the seventh week after the initial OMT treatment, mice received 8 training trials (60 s) with 15 min interval each day for 5 consecutive days. The test apparatus was a white iron pool (1.5 m in diameter and 0.6 m in height) containing 25 ± 2 °C water and was artificially divided into four quadrants. A cylindrical platform (40 cm in diameter and 6 cm in height) was hidden at a constant location beneath the surface of the water. The mouse was placed at one quadrant and swam 60 s freely searching for the platform. The escape latency was the time from mouse swimming to finding the platform. If the mouse could not find the platform, it would be guided to the platform and the escape latency was termed as 60 s. The data were collected using a camera equipped with Noldus Ethovision 8.5 software.

2.4. Immunohistochemical staining

After behavioral tests, mice were sacrificed on the eighth week. Mice were heart perfused with 4% paraformaldehyde and brains were harvested. Brains were sectioned coronally into 30 μm using a Leica CM1950. After blocking with 5% donkey serum, the slices were incubated with primary antibody solution overnight at 4 °C. After rinsing with PBS, slices were then incubated with secondary antibody solution for 2 h at room temperature and then mounted for visualization after another PBS rinse. The antibody information is listed as follow: anti-Aβ antibody (1:500, SANTA CRUZ, sc-28356), anti-GFAP antibody (1:500, Sigma, G6171), anti-NeuN monoclonal antibody (1:1000, Millipore, MABN140), Alexa Fluor 555 Donkey anti-mouse antibody (1:250, Life Technologies, A31570), Alexa Fluor 555 Donkey anti-rabbit antibody (1:250, Life Technologies, A31572) and Alexa Fluor 488 Donkey anti-mouse antibody (1:250, Life Technologies, A21202). Images were acquired using an Olympus FV1000 confocal laser scanning microscope equipped with 20× and 60× objectives.

2.5. Measurement of pro-inflammatory cytokines by ELISA

Mice brains were taken out after heart perfusion on the eighth week and dipped into liquid nitrogen immediately for frozen. The brains were then homogenized by a protease inhibitor cocktail (B14001, Selleck) and followed by ultrasonication for 10 min. Then the mixture was centrifugated at 2000 rpm for 8 min at 4 °C and the supernatant was collected for measurement of pro-inflammatory cytokines with ELISA kits (R&D systems) according to the manufacturer's instructions.

2.6. Statistics

Data are reported as mean ± SD. SPSS 24 (IBM) was used in the data analysis. Two-tailed unpaired *t*-test and ANOVA analyses with *post hoc* Tukey's multiple comparison test were applied in the group comparison. The difference was considerate statistically significant only when p value $< .05$.

3. Results

3.1. Oxymatrine significantly improved the cognitive abilities of AD mice during different behavioral tests

To test whether treatment of oxymatrine can attenuate the cognitive deficits of AD mice, eight-month-old hAPP AD mice were intraperitoneally injected with oxymatrine daily for 8 weeks (Fig. 1B). After the initial oxymatrine treatment, we performed open (and closed) field test (OFT), elevated plus maze test (EPM) and Morris water maze test (MWM) on the fifth, sixth and seventh week respectively (Fig. 1B). Wild type mice (WT mice), AD mice and AD mice treated with vehicle (AD + Veh mice) were used as control groups.

During the open/closed field test, AD mice and AD + Veh mice showed a significant reduction in the distance moved and the number of lattices crossing compared with WT mice (Fig. 2A and C). Although the performance of AD mice treated with oxymatrine (AD + OMT mice) did not recovery back to the WT mice level, they had a significant better performance compared with AD mice and AD + Veh mice (Fig. 2A and C). The result indicated that treatment of oxymatrine can significantly improve the locomotor activity of AD mice. During the elevated plus maze test, AD mice and AD + Veh mice spent more time in one side of the closed arm and seldom entered the open arms (Fig. 3A, B and C). When treated with oxymatrine, AD mice spent more time in both sides of the closed arm and explored more into the open arms (Fig. 3A, B and C). This indicated that treatment of oxymatrine can significantly decreased the anxiety level of AD mice. Morris water maze test is a classic behavioral model for testing the spatial learning and memory abilities. During the first two days of MWM, four groups of mice showed similar results in the escape latency (Fig. 4A). On the third and fourth testing days, WT mice significantly shortend the time for platform targeting while AD mice and AD + Veh mice still had a long escape latency (Fig. 4A). Quantitative analysis showed that OMT treatment significantly increased the time spent in the target quadrant (Fig. 4B and C) and the number of platforms crossing (Fig. 4B and D). The above behavioral tests clearly demonstrated that oxymatrine treatment can significantly attenuate the cognitive deficits of AD mice.

3.2. Oxymatrine reduced amyloid plaques in the neocortex and hippocampus of AD mice

The progressive accumulation of Aβ plaques is the main hallmark of Alzheimer's disease (Hardy and Higgins, 1992). $A\beta_{42}$ oligomers are the main component of senile plaques and exert neurotoxicity to the CNS, and eventually contribute to the damage of cognitive and memory (Hardy and Selkoe, 2002; Takahashi et al., 2017). To test whether the improvement of cognitive abilities of AD + OMT mice is associated with the elimination of Aβ, we sacrificed the mice on the eighth week after behavioral tests and immunochemically stained for Aβ. In the neocortex, Aβ plaques were totally absent in WT mice but can be clearly identified in the other three groups (Fig. 5A). However, the density of Aβ plaques was significantly reduced in AD + OMT mice compared with AD mice and AD + Veh mice (Fig. 5A and C). Similar results were obtained in the hippocampus (Fig. 5B and D). These results indicated

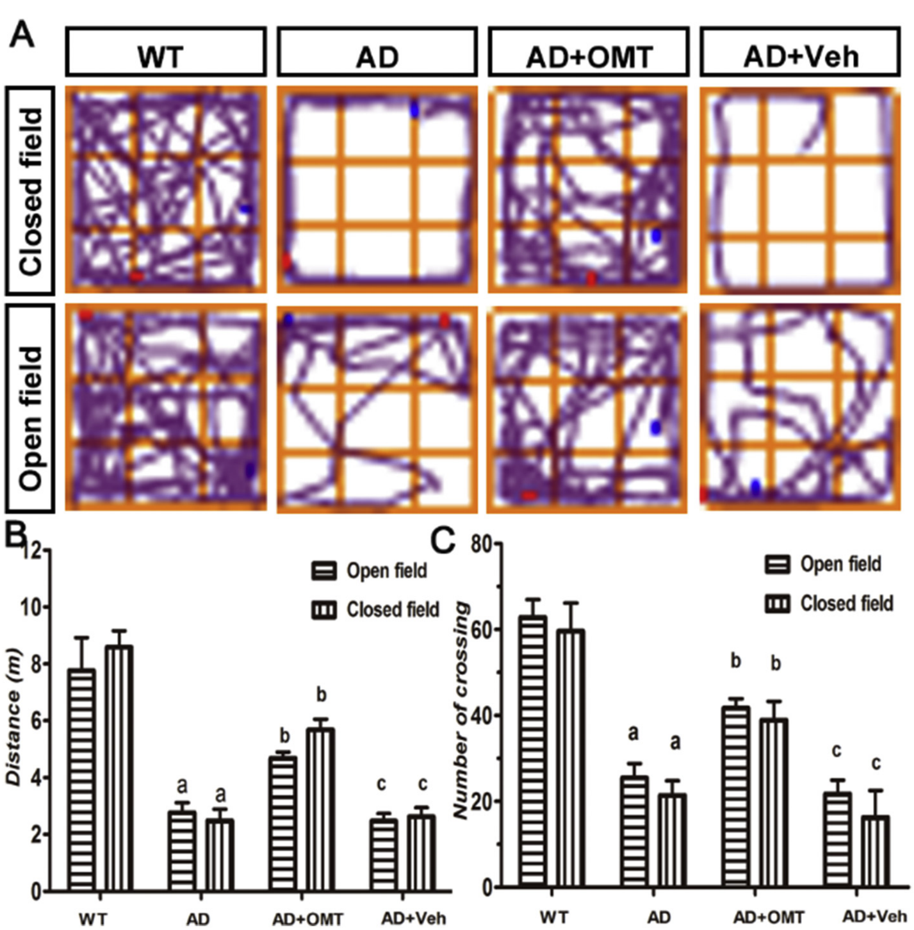

Fig. 2. Treatment of oxymatrine improved locomotor activity of AD mice. (A) Representative images showing that treatment of OMT increased locomotor activity of AD mice in the open/closed field tests. (B) Histograms of total distance moved by different treatment groups. (C) Histograms of number of lattices crossing recorded in different treatment groups. Data are presented as mean ± SD. $^{a}p < 0.05$ relative to the WT group. $^{b}p < 0.05$ relative to the AD group. $^{c}p < 0.05$ relative to the AD + OMT group. $n = 6$ mice for each column.

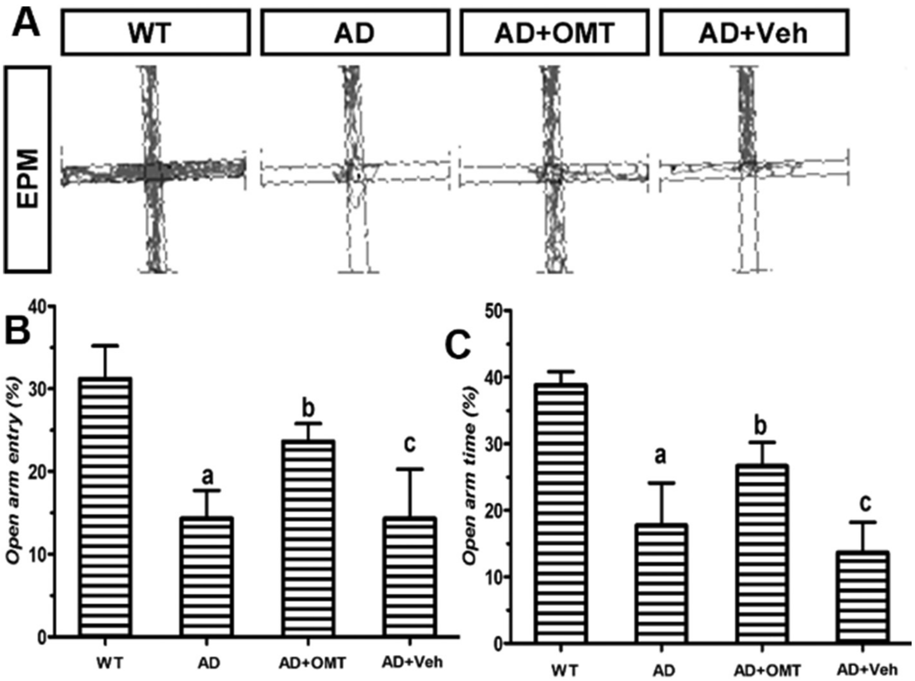

Fig. 3. Treatment of oxymatrine reduced the anxiety level of AD mice. (A) Representative images showing that treatment of OMT increased the activity in the open arms during the elevated plus maze test. (B) Percentage of open arm entry during the elevated plus maze test. (C) Percentage of open arm time during the elevated plus maze test. Data are presented as mean ± SD. $^{a}p < 0.05$ relative to the WT group. $^{b}p < 0.05$ relative to the AD group. $^{c}p < 0.05$ relative to the AD + OMT group. $n = 6$ mice for each column.

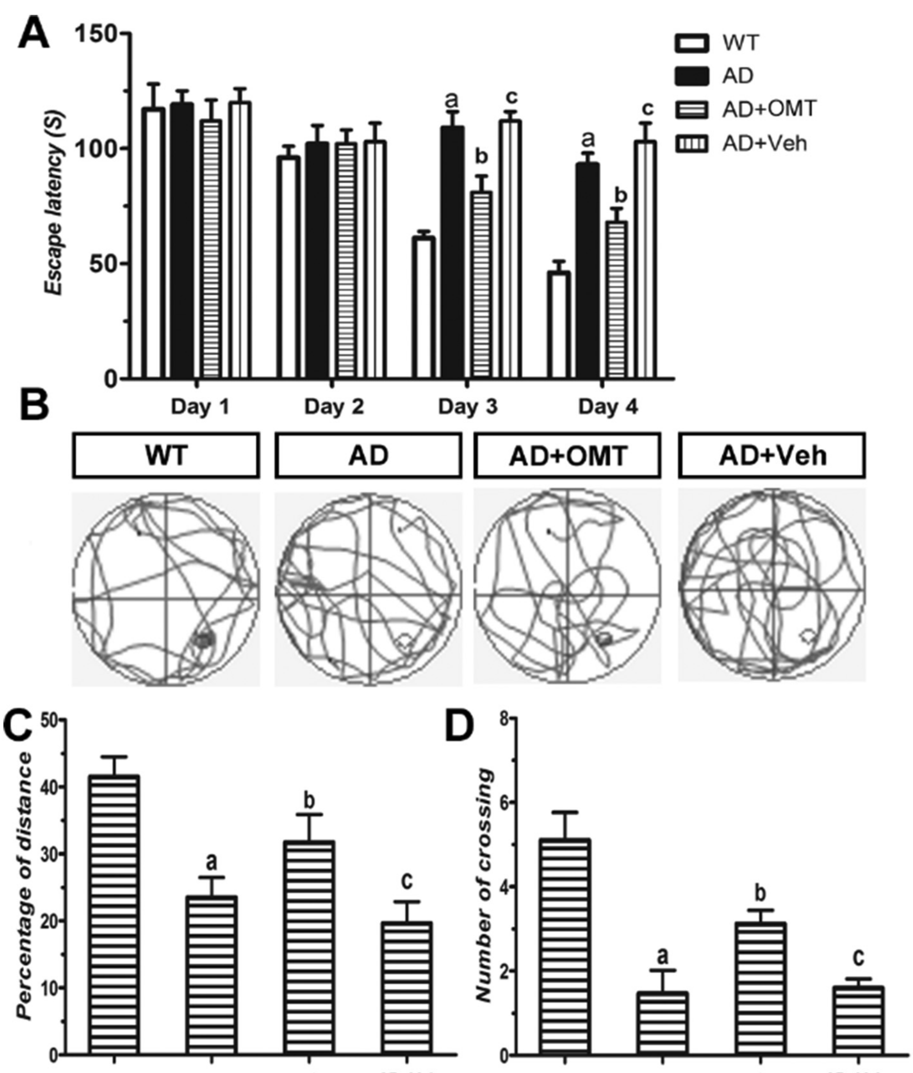

Fig. 4. Treatment of oxymatrine improved spatial learning and memory abilities of AD mice. (A) Histograms of escape latency on different days during Morris water maze. (B) Representative images showing that treatment of OMT increased learning and memory abilities of AD mice during Morris water maze. (C) Histograms showing percentage of distance in the target quadrant by different groups. (D) Histograms showing the number of platforms crossing by different groups. Data are presented as mean ± SD. $^{a}p < 0.05$ relative to the WT group. $^{b}p < 0.05$ relative to the AD group. $^{c}p < 0.05$ relative to the AD + OMT group. $n = 6$ mice for each column.

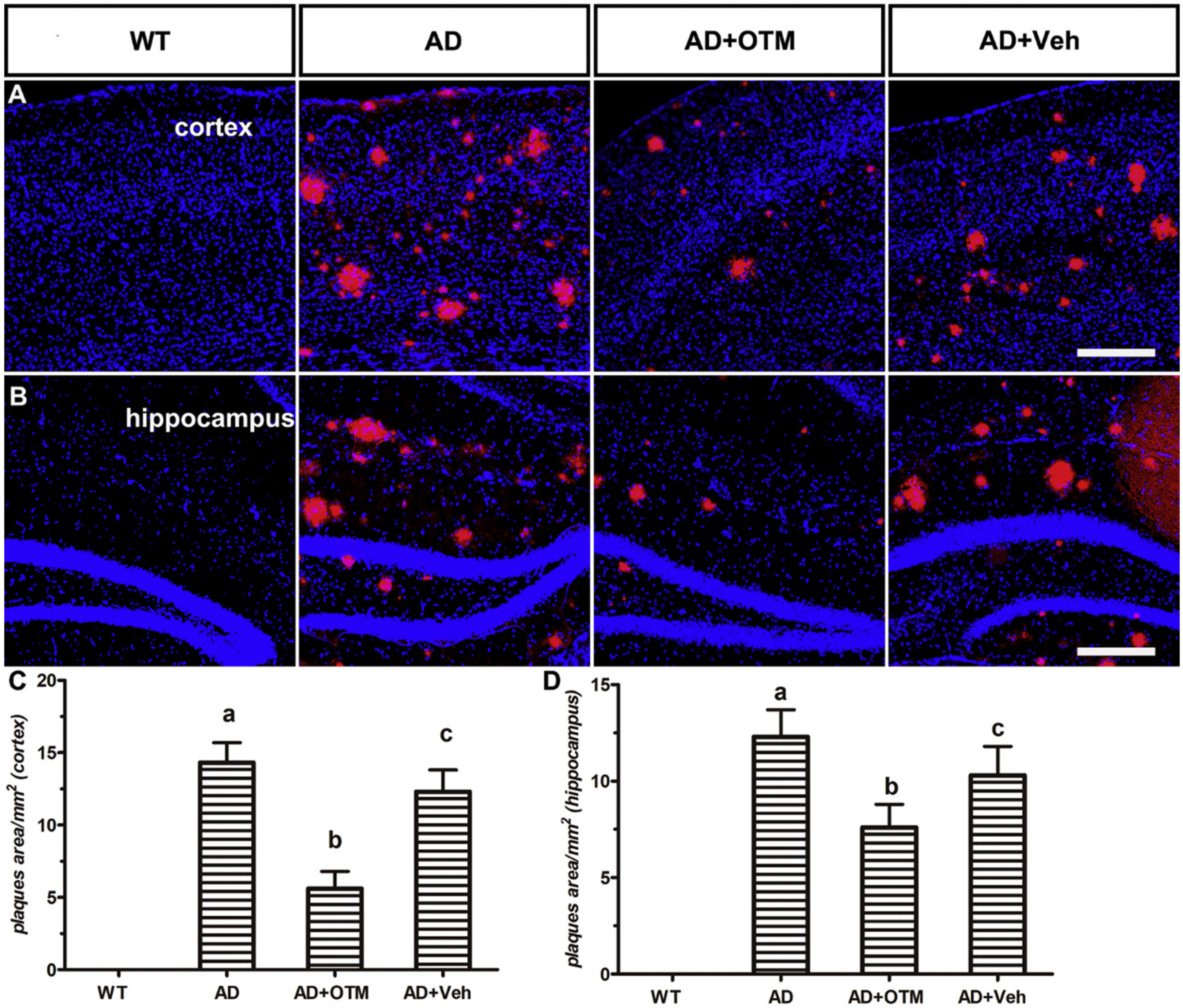

Fig. 5. Treatment of oxymatrine reduced the density of amyloid plaques in the neocortex and hippocampus. (A) Sample images indicating that OMT treatment reduced amyloid plaques in the neocortex of AD mice. Scale bar, 200 μm. (B) Sample images indicating that OMT treatment reduced amyloid plaques in the hippocampus of AD mice. Scale bar, 200 μm. (C and D) Quantitative analysis of the densities of amyloid plaques in the neocortex and in the hippocampus. Data are presented as mean ± SD. $^{a}p < 0.05$ relative to the WT group. $^{b}p < 0.05$ relative to the AD group. $^{c}p < 0.05$ relative to the AD + OMT group. $n = 20$ sections from 6 mice for each column.

that treatment of oxymatrine can effectively eliminate the aggregation of Aβ in AD mice.

3.3. Oxymatrine astrocyte clusters in the neocortex and hippocampus of AD mice

Neuroinflammation plays a central role in the development of Alzheimer's disease. The initial damage caused by Aβ or hyper-phosphorylated τ protein trigger immune response mediated by astrocytes and microglia and eventually lead to the pathological neuronal loss (Choi et al., 2016; Lim et al., 2013; Morales et al., 2013). Surrounding Aβ plaques, astrocytes are key elements in the clearance of extracellular Aβ (Lian et al., 2016; Liu et al., 2016). The clusters of astrocytes reflect pathological neuroinflammatory response and serve as another hallmark of AD (Bouvier and Murai, 2015). We stained Glial fibrillary acidic protein (GFAP) to detect the level of astrocyte clusters (Figs. 6 and 7). The GFAP signals in the neocortex and hippocampus of AD mice and AD + Veh mice were relatively strong compared with WT mice, indicating a high astrocyte clustering level in these mice (Figs. 6 and 7). However, when treated with oxymatrine, the astrocyte clustering level significantly reduced in both neocortex and hippocampus (Figs. 6 and 7). The above results clearly manifested that oxymatrine can effectively eliminate the densities of Aβ plaques and astrocytes clusters.

3.4. Oxymatrine significantly reduced pro-inflammatory cytokines in AD mice

Pro-inflammatory cytokines are largely involved in the initiation of chronic neuroinflammation caused by Aβ aggregation or hyper-phosphorylated τ protein (Patel et al., 2005; Smith et al., 2012). The level of cytokines maintains at high level in both AD patients and transgenic mice, which can be used as an index for evaluating AD progression and treatment efficacy (Cacabelos et al., 1994; Patel et al., 2005; Sutinen et al., 2012). Interleukin-6 (IL-6), IL-1β, IL-17A and tumor necrosis factor-α (TNF-α) were measured in the four groups of mice. Compared with WT mice, the other three groups showed significantly higher level of all four pro-inflammatory cytokines (Fig. 8). It should be noted that treatment of oxymatrine to AD mice significantly downregulated the level of the above cytokines (Fig. 8). The above result indicated that treatment of oxymatrine can reduce inflammatory response in the brains of AD mice.

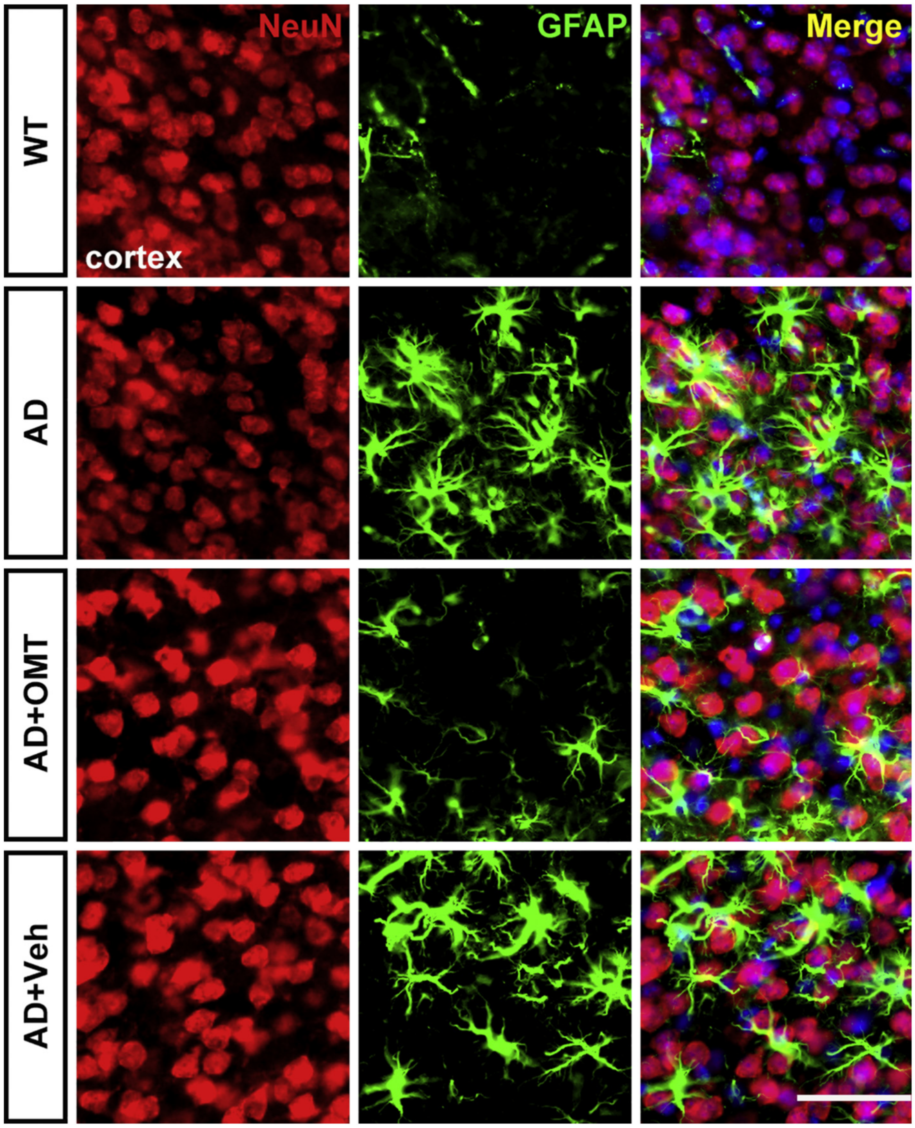

Fig. 6. Treatment of oxymatrine reduced astrocyte clusters in the neocortex of AD mice. Immunochemical images showing the staining of NeuN (left panel), GFAP (middle panel), and co-expression of NeuN, GFAP and DAPI (right panel). Scale bar, 100 μm.

4. Discussion

Alzheimer's disease (AD) is an age-related neurodegenerative disorder mainly affect people older than 65 years old and characterized by progressive learning and cognitive disorders (Alzheimer's Association, 2016). Many factors may take part in the pathogenesis of AD, such as genetics, brain trauma, glucose metabolism and even mental state (Ballard et al., 2011; Scheltens et al., 2016; Stern et al., 1995; Swaminathan and Jicha, 2014). The unclear pathologic mechanism of AD makes it still difficult to find an effective therapy for the disease. More and more studies have shown that neuroinflammation plays a central role in the pathogenesis of AD and the regulation of neuroinflammation may be a potential therapeutic strategy for the disease (Assefa et al., 2018; Morales et al., 2014; Sun et al., 2018). Oxymatrine is a plant-derived alkaloid and reported to have anti-viral, anti-fibrosis, anti-tumor and anti-inflammatory effects (Li et al., 2017; Liu et al., 2009; Lu et al., 2016; Wu et al., 2017; Zhao et al., 2018). The widely use of oxymatrine as an immunosuppressive agent makes it a possible candidate to treat AD.

In this study, we used hAPP AD mice to test whether treatment of oxymatrine can attenuate the damage of AD. As the typical AD model, hAPP transgenic mice exhibit age-dependent impairments in cognitive and memory abilities (Cheng et al., 2007; Palop et al., 2007, 2003; Palop and Mucke, 2010; Roberson et al., 2007). Our study found that treatment of oxymatrine significantly elevated the locomotor activity, the learning and memory abilities of hAPP mice during field tests, elevated plus maze tests and Morris water maze tests. Our result further confirmed previous work indicating that oxymatrine can attenuate behavioral deficits of Alzheimer's disease.

The existence of cortical Aβ plaques is the hall marker of AD. The senile plaques are mainly formed by $A\beta_{42}$ oligomers which are produced by the aberrant proteolytic cleavage of the amyloid precursor protein (APP) by β-secretase and γ-secretase (Hardy and Selkoe, 2002; Takahashi et al., 2017). Extra-cellular Aβ accumulation depends on the balance between its generation and clearance. Astrocytes are star-shaped and the most abundant type of glia cells in the CNS. Compelling studies have indicated that astrocytes take part in the clearance of Aβ from the brain parenchyma into the perivascular space across blood brain barrier and assist in the microglial phagocytosis of Aβ (Maresca et al., 2015; Sharma et al., 2012). However, in AD conditions, the existence of Aβ triggers the reactivation and dysfunction of astrocytes. In the other hand, reactive astrocytes facilitate Aβ aggregation by promoting the activity of β-secretase and γ-secretase (Cai et al., 2017; Guénette, 2003; Laurent et al., 2018). This vicious cycle between Aβ

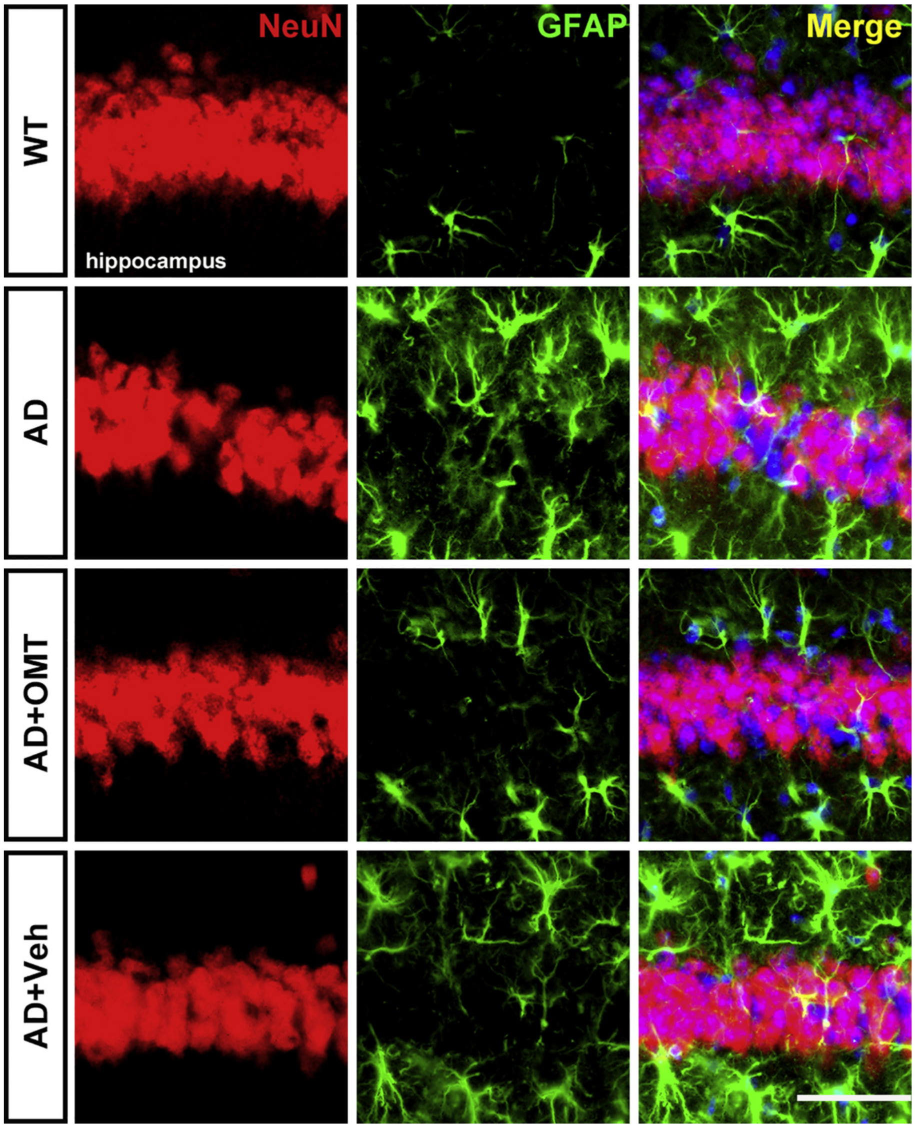

Fig. 7. Treatment of oxymatrine reduced astrocyte clusters in the hippocampus of AD mice. Immunochemical images showing the staining of NeuN (left panel), GFAP (middle panel), and co-expression of NeuN, GFAP and DAPI (right panel). Scale bar, 100 μm.

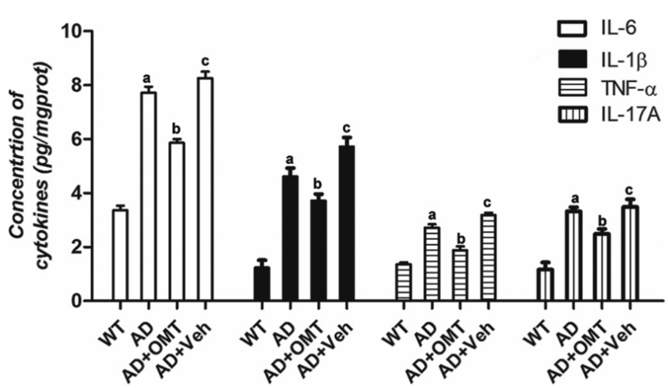

Fig. 8. Treatment of oxymatrine reduced the concentration of cytokines in AD mice. The concentration of IL-6, IL-1β, TNF-α and IL-17A of AD mice was significantly reduced when treated with OMT compared with AD mice and AD mice treated with vehicle. Data are presented as mean ± SD. $^{a}p < 0.05$ relative to the WT group. $^{b}p < 0.05$ relative to the AD group. $^{c}p < 0.05$ relative to the AD + OMT group. $n = 6$ samples from 6 mice for each column.

and astrocytes largely contribute to the development of AD. Similar to previous reports, hAPP mice displayed an elevation of cortical Aβ plaques and astrocyte clusters. Our treatment of oxymatrine to hAPP mice significantly reduced the level of both Aβ plaques and astrocyte clusters. These results indicated that oxymatrine can attenuate the development of AD in the cellular level.

Neuroinflammation plays a central role in the process of pathological neuronal loss which is the main cause of neurodegenerative diseases. Astrocytes and microglia are two main immunocytes in the central neural system (Morales et al., 2014). By sensing the initial injuring signals, astrocytes and microglia are gradually activated and then secrete a series of pro-inflammatory elements such as chemokines, cytokines and ROS, and eventually lead to the necroptosis or the deletion of harmful components (Bradt et al., 2000; Frautschy et al., 1992). However, in AD conditions, Aβ oligomers and phosphorylated τ aggregates induce additional immune response which eventually lead the inflammatory response from acute state into chronic state (Streit et al., 2004). In the CNS, Aβ and phosphorylated τ aggregates reactivate astrocytes and microglia, elevate the level of different cytokines (IL-6, IL-1β and TNF-α) and pro-inflammatory mediators (Cai et al., 2017; Newcombe et al., 2018). The upregulated cytokines impair the normal function of blood brain barrier and pathologically reactivate astrocytes in the central neural system through feedback loop (Cai et al., 2017; Newcombe et al., 2018). Because of the pathological contribution of cytokines to the development of AD, the expression levels of different cytokines may serve as signals for AD progression. Compared with the wild type mice, hAPP mice showed a significant upregulation of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α and IL-17A. After the treatment with oxymatrine, the level of cytokines in hAPP mice significantly reduced. Our results indicated that oxymatrine may attenuate the impairments of AD through cytokines related immunomodulatory pathways.

In this study, taking advantage of hAPP mice we clearly demonstrated that oxymatrine can attenuate the behavioral deficits of the model mice and downregulate the densities of Aβ plaques and astrocyte clusters. Our results also indicated that the anti-AD effects of oxymatrine may largely due to its involvement in the cytokines related inflammatory pathways. However, several obstacles still exist in the clinical usage of oxymatrine as an anti-AD drug. Firstly, more detailed pharmacological mechanisms (potential targets, signal pathways and pharma dynamics) of oxymatrine are needed to be illuminated. Secondly, the anti-AD effects of oxymatrine should be tested widely in other AD models. More importantly, strict clinical trials are needed to confirm the clinical effects of oxymatrine, since current therapeutic strategies (such as anti-Aβ, anti-inflammatories, statins and hormonal strategies) are miserable during different clinical trial phases.

5. Conclusion

Oxymatrine can significantly improve behavioral performance of AD mice and reduce the densities of Aβ plaques and astrocyte clusters. Besides, these effects of oxymatrine may largely due to the its role in the downregulation of pro-inflammatory cytokines.

Competing interests

The authors declare no competing financial interests.

Author contributions

Y. C. and H. L. designed the study. Y. C. performed the experiments and collected the data. Z.Q. performed behavioral tests. B. Q. performed immunohistochemistry. Z. L. and T. H. analyzed the experimental data. Y. C. and H. L. wrote the manuscript.

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