Atorvastatin attenuates the production of IL-1β, IL-6, and TNF-α in the hippocampus of an amyloid β1-42-induced rat model of Alzheimer’s disease

Yuan-Yuan Zhang1,2,*
Yu-Chen Fan3,4,*
Min Wang1,2
Dong Wang1,2
Xiao-Hong Li1,2

1Department of Neurology, Jinan Central Hospital affiliated to Shandong University, Jinan, People’s Republic of China; 2School of Medicine, Shandong University, Jinan, People’s Republic of China; 3Department of Neurology, Jinan Central Hospital affiliated to Shandong University, Jinan, People’s Republic of China; 4Department of Hepatology, Qilu Hospital of Shandong University, Jinan, People’s Republic of China

*Yuan-Yuan Zhang and Yu-Chen Fan contributed equally to this work

Background and aim: Amyloid-beta (Aβ) peptide is reported to initiate flexible inflammation in the hippocampus of the human brain in Alzheimer’s disease (AD). The present study aimed to investigate the possible effects of atorvastatin on the production of inflammation cytokines in the hippocampus and the potential impacts on behavioral ability, in an AD model.

Methods: We firstly established AD rat models using intracerebroventricular injection of Aβ1-42. A Morris water maze was also performed to determine the spatial learning and memory ability in the AD models. Intracellular staining of interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha was determined using immunohistochemical staining at 6 hours and day 7 after the injection of Aβ.

Results: The escape latency of the atorvastatin-treated AD group (5 mg/kg/d) was significantly shorter than that of AD group on day 3 (41 ± 1.05 seconds versus 47 ± 1.05 seconds, P < 0.01) and day 4 (34 ± 1.25 seconds versus 43 ± 1.01 seconds, P < 0.01) after the beginning of the training. Furthermore, the atorvastatin-treated AD group displayed a significant higher mean number of annulus crossings than did the AD group (2.9 ± 0.5 versus 2.4 ± 0.9, P < 0.05). Fewer injured nerve cells and proliferated glial cells were also demonstrated in the atorvastatin-treated AD group than in the AD group. Of great importance, we demonstrated that IL-1β, IL-6, and tumor necrosis factor alpha were significantly decreased in the atorvastatin-treated AD group than that in the AD group.

Conclusion: Atorvastatin might attenuate the damage of nerve cells and improve learning and memory ability by inhibiting inflammatory response in the progression of AD.

Keywords: amyloid-beta, inflammation, interleukin-1β, interleukin-6, tumor necrosis factor

Introduction
Alzheimer’s disease (AD) is a progressive neurodegenerative condition which is characterized by the extraneuronal deposits of beta-amloid (Aβ) fibrils and intraneuronal accumulations of hyperphosphorylated tau protein. In the progression of AD, it has been demonstrated that accumulation and aggregation of Aβ peptide in the hippocampus of the brain usually results in the activation of glial cells which, in turn, initiates a neuroinflammatory response, involving reactive oxygen intermediates and inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF-α). In addition, soluble Aβ is also reported to induce inflammation. Experimental and clinical data demonstrate that cholesterol is a possible factor in the etiology of AD. Cholesterol-lowering agents, particularly 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (also known as statins), have been reported to be capable of increasing the ratio of alpha to beta secretase activity and then increasing the concentrations of extracellular Aβ. Furthermore, accumulating clinical
epidemiological data from various populations has showed that statin users have had low incidence of AD.8–11 This low incidence of AD in statins users was previously thought to be attributed to the cholesterol-lowering activity of statins. Currently, the non-cholesterol-lowering actions of statins, including their action on nitric oxide, platelet adhesion, and anti-inflammatory action, have been reported.12 In addition, statins can also increase endothelial nitric oxide synthase (eNOS), and reduce endothelin-1, thereby resulting in relaxation of vascular smooth muscles and leading to vasodilatation.13,14 Interestingly, inflammatory response has been demonstrated to be involved in the activation of plaque-associated microglia in the progression of AD. The activated microglias are also capable of releasing a wide range of proinflammatory factors and then exacerbating neuronal death.15,16 Therefore, it is speculated that the anti-inflammatory activity of statins might partly contribute to the low incidence of AD in statins users.

Simvastatin has been demonstrated to reduce levels of Aβ42 and Aβ40 in vitro and in vivo.17 Atorvastatin is a member of the statin family, which is mainly prescribed for the treatment of hypercholesterolemia and has been shown to have potential beneficial therapeutic anti-inflammatory effects.18 Therefore, we speculated that atorvastatin might attenuate learning and memory ability in an AD model, through the inhibition of inflammatory response. In this present study, we then mainly intended to investigate the possible effects of atorvastatin on hippocampal inflammation and behavioral ability, including learning and memory, in an AD rat model induced by Aβ1-42.

Materials and methods

Animal treatment

A total of 60 adult male Wistar rats (250–350 g), purchased from the Experimental Animal Center of Shandong University (Shandong, People’s Republic of China), were included in this study. The rats were housed in groups of five, had free access to food and water, and were maintained under veterinary supervision at an ambient temperature of 22°C–23°C, under a 12:12 hour light/dark cycle. All the rats received human care, and the experiment was approved by the Animal Care and Use Committee of Shandong University, and every effort was made to minimize stress to the animals. The rats were adapted for 2 weeks to the above conditions prior to the present experiment.

All the rats were randomly classified into four groups: the control group (Group 1); the AD group (Group 2), the atorvastatin control group (Group 3); and the atorvastatin-treated AD group (Group 4). A Morris water maze (MWM) was performed at the beginning of the study and there was no significant difference among the four groups. Groups 3 and 4 were treated with atorvastatin for 3 weeks, and the other two groups were treated with saline. After 3 weeks, Groups 2 and 4 were injected intracerebroventricularly with Aβ1-42, and the other two groups with saline. Brain tissue was obtained for pathological observation and intracellular staining at 6 hours (n = 5) and on day 7 after Aβ1-42 injection (n = 5). An MWM (n = 5) was performed, to identify learning and memory ability, on day 7 after the establishment of the AD model.

Materials

Aβ1-42 (Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile saline at a concentration of 2 g/L and stored at −20°C. To obtain the neurotoxic form of Aβ1-42, the peptide solution was placed in an incubator at 37°C for 7 days. Rabbit anti-IL-1β, goat anti-IL-6, and goat anti-TNF-α polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Biotin conjugated anti-rabbit or anti-goat secondary antibody was purchased from Beijing Zhongshan Biotechnology Co., Ltd (Beijing, People’s Republic of China).

Induction of cognitive dysfunction by injection of Aβ1-42 and treatment with atorvastatin

The AD model was induced with the injection of Aβ1-42 into the ventricle (2.5 mm posterior and 0.5 mm lateral to the bregma). A microsyringe with a stainless steel needle was used for this experiment. Rats were anesthetized with sodium pentobarbital (40 mg/kg) and placed in a stereotaxic apparatus. Then, 5 mL of peptide (10 μg) or vehicle control was delivered gradually within 3 minutes, and the syringe was kept at the site for 2 minutes following the injection.

Atorvastatin, 5 mg/kg per day (Lipitor®; Pfizer Inc, New York, NY, USA) and saline (2 mL) were mixed with regular food via intragastric administration, and rats were examined daily and weighed at intervals throughout the study. The dose of atorvastatin was decided on the basis of previous findings,19,20 this was higher than the human dose (1.1 mg/kg per day) recommended for the treatment of hypercholesterolemia21 but was consistent with the pharmacokinetic data reporting a higher metabolic rate of the drug in rodents.22

Morris water maze

All the rats were trained on a MWM test on day 7 after the injection of Aβ1-42. The apparatus consisted of a large circular pool, approximately 1.0 meter in diameter, containing water, at around 25°C, that was made opaque by
the addition of milk to help hide the submerged platform. The movements of the animal in the tank were monitored with a video tracking system (EthoVision, Noldus Information Technology, Wageningen, The Netherlands).

Reference memory test
For each training trial, the rat was put into the pool at one of the five positions, the sequence of the positions being selected randomly. The platform was located a constant position throughout the test period, in the middle of one quadrant and equidistant from the center and edge of the pool. In each training session, the latency to escape onto the hidden platform was recorded. If the rat found the platform, it was allowed to remain there for 10 seconds and was then returned to its home cage. If the rat was unable to find the platform within 60 seconds, the training was terminated, and a maximum score of 60 seconds was assigned. Training was conducted for four consecutive days, four times per day, from day 7 to 10, after injection of Aβ1-42.

Probe test
On day 11 after injection of Aβ1-42, a single probe trial was conducted. The platform was removed from the pool, and each rat was allowed to swim for 60 seconds in the maze. The number of times the rat crossed the annulus where the platform had been located was recorded.

Working memory (repeated acquisition) test
A working memory test was conducted on three consecutive days from day 11 to 13 and consisted of five trials per day. The working memory test was procedurally similar to reference memory test except that the platform location was changed daily. The first trial of the day was an informative sample trial in which the rat was allowed to swim to the platform in its new location. Spatial working memory was regarded as the mean escape latency of the second to fifth trials.²³

Pathological features
Cardiovascular perfusion fixation was carried out after behavioral test. The rats, anesthetized by celiac injection of 10% chloral hydrate (3 mL/kg), were perfused with 100 mL chilled 0.9% saline first and then fixed with 150 mL paraformaldehyde (4%, 4°C). The brains were taken out quickly on an icy table and then put into a paraformaldehyde solution (4%) for fixation. Sequential tissue sections (5 μm thick) were made along the coronal profile, after fixation and paraffin embedment. Hippocampal sections were put on clean slides treated with poly-D-lysine and baked for 2 hours at 60°C, then kept in a refrigerator at 4°C, for hematoxylin and eosin (HE) staining. Structural features were observed at 6 hours and on day 7, after Aβ1-42 injection.

Immunohistochemistry procedure
IL-1β, IL-6, and TNF-α proteins expressed in the brain tissue were detected by immunostaining, according to the standard streptavidin-biotin-peroxidase technique. Briefly, the paraffin wax–embedded sections were dewaxed and incubated with 3% H₂O₂ for 5 minutes, to inactivate the native peroxidase. After rinsing with distilled water, the sections were kept in phosphate-buffered saline (PBS) for 5 minutes and incubated with primary antibody diluent for 10 minutes. After incubation with primary antibody diluent, the sections were rinsed three times for five minutes with PBS and then incubated with secondary antibody diluent for 30 minutes. The sections were rinsed three times for 3 minutes with PBS before incubation with streptavidin-HRP complex and reaction was with 3,3′-diaminobenzidine (DAB) solution for 10 minutes after being rinsed another three times for 5 minutes with PBS. The sections were enveloped with gelatin and observed under the microscope. For semiquantitative immunohistochemistry, the number of positive cells was counted by an investigator blinded to the experimental groups. Under the microscope, the counting area for hippocampus CA1 area of each section was exactly the size of a complete field at ×200 magnification. Only the positive cells inside the pyramidal layer were counted. An average of the serial four slices was taken from each individual rat. Four rats were taken from each group.

Statistical analysis
Data was expressed as mean ± standard derivation (SD) for each individual. Statistical analysis of the data for multiple comparisons was performed by one way analysis of variance (ANOVA) with a post hoc test. A level of P < 0.05 was accepted as statistically significant.

Results
Effects of atorvastatin on learning and memory
The spatial learning and memory ability of rat models were determined using the following parameters: escape latency onto a hidden platform, mean number of annulus crossings, and mean escape latency. We obviously found that the escape latencies of the AD rat model after 2–4 days of training were significantly longer than the two groups without AD. Figure 1A demonstrates
that the escape latency of the atorvastatin-treated AD group was significantly shorter than AD group on day 3 (41 ± 1.05 seconds versus 47 ± 1.05 seconds, \( P < 0.01 \)) and day 4 (34 ± 1.25 seconds versus the beginning of the training 43 ± 1.01 seconds, \( P < 0.01 \)). In the 60 second probe test, the mean number of annulus crossings of the atorvastatin-treated AD group showed significant higher times of annulus crossing than did the AD group (2.9 ± 0.5 versus 2.4 ± 0.9, \( P < 0.05 \)) (Figure 1B). In Figure 1C, working memory was assessed as the mean escape latency of the second to the fifth trial for 3 days. Of great interest, the atorvastatin-treated AD group showed a significant decrease in mean escape latency from the AD group (12.3 ± 3 seconds versus 15.8 ± 4.2 seconds, \( P < 0.05 \)). These results strongly suggested that atorvastatin might be capable of attenuating the impaired spatial learning and memory in AD rat models.

**Effects of atorvastatin on pathological changes in rats’ hippocampus**

In this present study, HE staining was used for identifying the different macroscopic pathological features in the hippocampus of AD rat models. On day 7 after Aβ1-42 injection, the cellular nuclei were clear and homogeneously stained in the groups without atorvastatin treatment (Figure 2A and C). Furthermore, sparsely formed cells and deeply stained nuclei were obviously demonstrated in the AD group (Figure 2B). However, in the atorvastatin-treated AD group (Figure 2D), the neurons and the nuclei just looked the same as in the control group without Aβ1-42 injection.

**Effects of atorvastatin on the expression of proinflammatory factors**

To investigate the effects of atorvastatin on hippocampal inflammation, we determined the intracellular staining of certain proinflammatory factors, including IL-1\( \beta \), IL-6, and TNF-\( \alpha \), using an immunohistochemical method, at 6 hours and 7 days after the injection of Aβ. Generally, intracellular staining of IL-1\( \beta \), IL-6, and TNF-\( \alpha \) was mostly located in the cell plasma. The results showed that there was no obvious staining of IL-1\( \beta \), IL-6, or TNF-\( \alpha \) in the hippocampus of the control group (Figures 3A, 4A, and 5A). However, intracellular

---

**Figure 1** Atorvastatin improved the spatial learning and memory of rats impaired by Aβ1-42, using the Morris water maze test. (A) Escape latency onto a hidden platform; (B) probe test; and (C) working memory.

**Abbreviations:** Aβ, amyloid-beta (peptide); AD, Alzheimer’s disease; S, second.
staining of IL-1β, IL-6, and TNF-α were increased significantly (Figures 3C, 4C, and 5C) in the Aβ-treated group over that in the control and atorvastatin control groups. There were significant differences in the intracellular staining of IL-1β, IL-6, and TNF-α in the atorvastatin control group and the control group (Figures 3B, 4B, 5B and 3A, 4A, 5A). Of great importance, we demonstrated that IL-1β, IL-6, and TNF-α were significantly decreased in the atorvastatin-treated AD group than that in the AD group (Figures 3C, 4C, 5C, and 3D, 4D, 5D).

**Discussion**

The clinical data demonstrated that statins might reduce the risk rate for the incidence of AD. Meanwhile, the cholesterol-lowering activity and anti-inflammatory ability of the statin family have been considered to provide rationale for this hypothesis. In this present study, we firstly established the AD rat model using Aβ1-42 injection and then aimed to investigate the possible effects of atorvastatin on the inflammation and on behavioral ability, including learning and memory. Our results demonstrated that the escape latency of the atorvastatin-treated AD group was significantly shorter than in the AD group on the third and fourth day after the beginning of the training. Furthermore, the atorvastatin-treated AD group displayed a significant higher mean number of annulus crossings than did the AD group. Fewer injured nerve cells and proliferated glial cells were also found in the atorvastatin-treated AD group than in the AD group. Of great importance, we demonstrated that IL-1β, IL-6, and TNF-α were significantly decreased in the atorvastatin-treated AD group than that in the AD group. Our results strongly suggested that atorvastatin might attenuate the damage of nerve cells and improve learning and memory ability by inhibiting inflammatory response in the progression of AD.

In our present study, we found that the intracellular expressions of IL-1β, IL-6, and TNF-α was significantly increased after the injection of Aβ. Previous studies also demonstrated that increased inflammatory components contributed to the progression of AD. It is therefore hypothesized that inflammatory components could be involved in the activation of plaque-associated microglia. It was also demonstrated that inflammation might act to augment disease progression via its ability to stimulate neuronal Aβ production in the progression of AD, and to facilitate amyloid deposition. However, the exact mechanism underlying the inflammatory response action on the progression of AD is still obscure and needs to be further studied.

The hypothesis that statin treatment may be one independent factor in the incidence of AD has been strongly suggested. Wolozin et al performed a cross-sectional design and reported that statin therapy provided a...
our results showed that atorvastatin could attenuate the inflammatory response in the brain. In future work, better quantitative assays are necessary such as enzyme-linked immunosorbent assay (ELISA) to support this hypothesis. However, the exact molecular mechanism underlying the statin association with AD progression must be identified. The effects of atorvastatin treatment seemed dramatic and were surprising. Perhaps the dramatic differences come from the small number of rats, selection bias, the strains of the rats, and so on. However, the anti-inflammatory role of atorvastatin provided a potential therapy for the prevention of AD and this issue must be studied in future research.

There are some limitations in this present study. First, we only observed the positive staining of inflammatory cytokines in the hippocampus of an AD model. Other cells that stain positive for IL-1β, IL-6, and TNF-α must be identified using markers such as Iba-1 for microglia or glial fibrillary acidic protein (GFAP) for astrocytes. Second, we performed immunohistological staining to determine the presence of an inflammatory response in the brain. In future work, better quantitative assays are necessary such as enzyme-linked...
immunosorbent assay (ELISA), Western blot, or reverse transcription polymerase chain reaction (RT-PCR). However, our results still provide strong evidence that atorvastatin might attenuate the damage of nerve cells and improve learning and memory ability by inhibiting inflammatory response in the progression of AD.

**Conclusion**

Our present study aimed to investigate the possible effects of atorvastatin on inflammation and on behavioral ability, including learning and memory. Our results firstly demonstrated improved learning and memory ability under the treatment of atorvastatin, using AD rat models. We then found that IL-1β, IL-6, and TNF-α were significantly decreased in the atorvastatin-treated AD group than that in the AD group. Our results strongly suggested that atorvastatin might attenuate the damage of nerve cells and improve learning and memory ability, by inhibiting inflammatory response in the progression of AD. The potential therapeutic role of atorvastatin in the prevention of AD should be studied in future research.

**Acknowledgment**

This work was supported by grants from National Natural Science Foundation Shandong Province (Y2008C103) and Jinan Planning Project of Science and Technology (200807032).

**Disclosure**

The authors declare that they have no competing financial interests.

**References**


