

Encapsulating Ascorbic Acid in Chitosan Nanoparticles Enhances Its Neuroprotective Properties in a Rat Model of Alzheimer's Disease

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Abstract

Background: In recent years, scientists have discovered that nanoparticles can effectively aid in the treatment of neurodegenerative diseases. This study aimed to explore the effects of ascorbic acid (AA) encapsulated in chitosan nanoparticles on neuroprotection in a rat model of Alzheimer's disease. We employed biochemical, molecular, histological, and behavioral methods to achieve this purpose.

Methods: Alzheimer's disease was induced in the rats through an intracerebroventricular injection of amyloid beta ($A\beta$)₁₋₄₂. After the induction, the animals received either AA or nano-AA at a dosage of 100 mg/kg orally for one month.

Results: Both treatments effectively prevented the decrease in superoxide dismutase activity and the increase in malondialdehyde levels in the hippocampus of the $A\beta$ -injected rats. In addition, both AA and nano-AA inhibited the elevation of monocyte chemoattractant protein-1 and beta-secretase-1 expression. They also reduced amyloid plaque deposition and neuronal death in the hippocampal cornu ammonis 1 area. Moreover, the treatments led to improvements in spatial learning and memory. AA and nano-AA prevented the rise in indoleamine 2,3-dioxygenase-1 expression in the hippocampus, reduced kynurenine levels, and increased serotonin levels. Notably, nano-AA demonstrated greater effectiveness than AA across all observed factors, except for memory improvement.

Conclusion: In conclusion, encapsulating AA in chitosan nanoparticles enhances the antioxidant's ability to reduce oxidative stress and inflammation, improve histological damage, and regulate serotonin metabolism in the hippocampus of rats modeled for Alzheimer's disease.

Keywords: Ascorbic acid, Chitosan, Amyloid beta peptide, Serotonin, Hippocampus, Spatial learning

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia. Biologically, it is characterized by the buildup of amyloid- β ($A\beta$) outside of neurons and the presence of neurofibrillary tangles inside neurons, which consist of aggregated hyperphosphorylated tau protein. Specifically, $A\beta$ ₁₋₄₂,

produced by β -site amyloid precursor protein cleaving enzyme 1 (BACE1) and gamma-secretase, plays a role in the hyperphosphorylation of tau. Research has shown that the deposition of A β is necessary for the progression of tau pathology, and A β can also lead to dysfunctions in synaptic transmission independently of tau, resulting in cognitive impairment. A β disrupts the transmission of neurotransmitters such as acetylcholine, glutamate, γ -aminobutyric acid, dopamine, and serotonin in various brain regions, including the hippocampus, which is crucial for cognitive function.¹⁻³

Evidence shows that A β oligomers significantly contribute to oxidative stress and neuroinflammation in the brain. A β oligomers generate reactive oxygen species (ROS) that can damage proteins, lipids, and DNA. Additionally, A β promotes synaptotoxicity and neurotoxicity by activating astrocytes and microglia.⁴ Finding effective therapies for AD remains challenging for researchers. Despite progress in this field, we still lack a treatment that can effectively stop the disease from advancing. Because of A β 's crucial role in AD development and progression, targeting A β production and improving its clearance are key therapeutic goals.⁵ In this context, antioxidants have attracted considerable interest from researchers.⁶

Ascorbic acid (AA; C₆H₈O₆) is a water-soluble organic compound that is commonly found in fruits and vegetables. It is a powerful antioxidant that scavenges ROS and inhibits pro-inflammatory cytokines.^{7, 8} Studies indicate that patients with AD have lower levels of AA in their plasma compared to healthy individuals.⁹ Research has demonstrated that AA can prevent the aggregation of A β ₁₋₄₂¹⁰, and there are also reports of its protective effects in animal models of AD.^{11, 12} AA enters the brain via the choroid plexus but cannot cross the blood-brain barrier (BBB) due to the tight junctions between endothelial cells, which restrict paracellular transport.¹³

In recent years, nanoparticles have emerged as promising tools for treating chronic diseases of the central nervous system (CNS) due to their ability to cross the BBB and provide controlled drug release.¹⁴ Among these, chitosan-based nanoparticles are recognized as biodegradable yet stable carriers for delivering medications targeting the CNS. These nanoparticles possess several beneficial characteristics, including biocompatibility, sustained drug release, low toxicity, low immunogenicity, flexibility in surface modification, and antibacterial activity. The degradation products of chitosan nanoparticles are non-toxic, non-immunogenic, and non-carcinogenic. Remarkably, chitosan itself has a very high lethal dose 50 (LD₅₀) value, comparable to that of common substances like salt or sugar. Additionally, chitosan nanoparticles can be produced easily and rapidly, with both material and production costs being low.^{15, 16}

This study aimed to investigate how the loading of AA in chitosan nanoparticles affects its neuroprotection in a rat model of AD. To achieve this, we compared the effects of AA alone and chitosan nanoparticles containing AA on various parameters, including oxidative stress, neuroinflammation, amyloid plaque deposition, neuronal death, tryptophan metabolism in the hippocampus, and memory changes induced by A β ₁₋₄₂.

2. Materials and Methods

2.1. Synthesis and characterization of nanoparticle

AA (0.176 kDa), chitosan (100 kDa, 75% deacetylated), and sodium tripolyphosphate (TPP) (367.86 kDa) were purchased from Sigma-Aldrich (USA). Chitosan served as a carrier, while TPP acted as a crosslinking agent. Chitosan powder was dissolved in 1% acetic acid at a concentration of 1 mg/ml. Next, 10 mg of AA was dissolved in distilled water at a concentration of 1 mg/ml and

added to the chitosan solution. A TPP solution (0.2 mg/ml, prepared in distilled water) was then added dropwise to the mixture. After one hour, nanoparticles formed and were centrifuged at 10,000 rpm for one hour to separate them. Formulation trials were carried out 3 times to optimize the nanoparticle characteristics. The resulting precipitate was dissolved in water and stored at -70 °C for future use.

The morphology of the nanoparticle was analyzed using field-emission scanning electron microscopy (FESEM), and its size was measured using dynamic light scattering (DLS).

2.2. Animals and experimental design

Sixty male Wistar rats, each weighing between 200 and 220 g, were maintained under standard laboratory conditions, which included a temperature variation of 24 ± 1 °C and a 12-hour light/dark cycle, with unlimited access to food and water.

The practices for animal handling and experimental methods strictly follow the ARRIVE guidelines.¹⁸ Ethics approval was obtained by the Animal Care and Use Committee at Islamic Azad University - Science and Research Branch (Approval number: IR.IAU.SRB.REC.1402.338).

The rats were divided into six groups, with ten rats in each group:

1. Control group: These animals did not undergo surgery and did not receive any treatment.
2. PBS group: During stereotaxic surgery, these rats received phosphate-buffered saline (PBS) intracerebroventricularly (ICV) and were given distilled water (the solvent for AA) by gavage for one consecutive month.
3. A β group: During stereotaxic surgery, these rats received A β_{1-42} at a dose of 10 μ g per rat via ICV injection and were given distilled water by gavage for one consecutive month.¹⁹ A β_{1-42}

(Sigma-Aldrich, USA) was dissolved in 0.1 M sterile PBS at a concentration of 2.5 µg/µl, and 2 µl of this solution was injected into each lateral ventricle.

4. Aβ.C group: Following ICV injection of Aβ₁₋₄₂, these rats received chitosan (200 mg/kg) by gavage daily for one consecutive month.²⁰

5. Aβ.AA group: After ICV injection of Aβ₁₋₄₂, these rats were given AA (100 mg/kg) by gavage for one consecutive month.¹²

6. Aβ.nAA group: Following ICV injection of Aβ₁₋₄₂, these rats received nano AA (100 mg/kg) by gavage for one consecutive month.¹²

On Day 0, stereotaxic surgery was performed. One day later, treatments with distilled water, chitosan, AA, or nano AA were started and continued for one month. Spatial learning and memory were assessed using the Morris water maze (MWM) test from days 26 to 30. At the end of this treatment period, rats were anesthetized with ketamine and xylazine²², and their hippocampi were removed for analysis. The hippocampi from five rats were used to measure levels of malondialdehyde (MDA), serotonin, kynurenine, and superoxide dismutase (SOD) activity. The right hippocampus from another five rats was used to examine the expression of the genes BACE1, indoleamine 2,3-dioxygenase 1 (IDO1), and monocyte chemoattractant protein-1 (MCP-1) using real-time polymerase chain reaction (PCR). Meanwhile, the left hippocampus of these animals was analyzed for neuronal death through Nissl staining and for amyloid plaque deposition using thioflavin staining.

2.3. Stereotactic surgery

Rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg). The coordinates for injecting Aβ₁₋₄₂ (5 µg/2 µl) or PBS (2 µl) were chosen based on brain stereotaxic coordinates.²³

The specific injection site coordinates were: anteroposterior (AP): -0.8 mm, lateral (L): ±1.5 mm,

and vertical (V): 3.8 mm. Injections were performed using a Hamilton microsyringe (Hamilton, USA) at a rate of 1 µl every 2 min into both lateral ventricles.

2.4. MWM test

The MWM test is a commonly used method for assessing animal learning and memory. This procedure consists of two phases. The first phase lasted four days, from days 26 to 29, during which each animal swam in a water tank for four 90 s periods per day. The goal was for the animal to find a hidden platform located 2 cm below the water's surface. The average time (s) that each rat took to locate the platform each day was recorded and served as a measure of learning. On day 30, the platform was removed, and a test known as the probe trial was conducted. In this trial, each animal swam in the tank for 60 s without the platform. The time (s) that each rat spent in the target quadrant was recorded and served as an indicator of memory. The movements of the animals in the tank were recorded using a video tracking system positioned above the maze apparatus.²⁴ The data was then analyzed by EthoVision software (Noldus, Netherlands).

2.5. Biochemical assessment

For biochemical studies, hippocampi were homogenized in a Tris buffer at pH 7.5. The samples were then centrifuged at 12,000 g for 20 min, and the protein concentration in the supernatant was measured using the Lowry method.²⁵

MDA levels in the hippocampus were assessed using the Nalondi™ Lipid Peroxidation Assay Kit (Navand Salamat, Iran), following the provided instructions. In summary, MDA reacts with thiobarbituric acid when heated, producing a pink compound. The absorbance of this compound is measured at 550 nm.

SOD activity was evaluated using the Nasdox™ Assay Kit (Navand Salamat, Iran). This kit works by inhibiting the oxidation of pyrogallol and measuring its oxidation half-life at a specific concentration. The SOD activity in an unknown sample was determined at 405 nm by comparing the inhibition of pyrogallol oxidation over time with that of a SOD control concentration.

Serotonin and kynurenine levels were assessed using ELISA kits (MyBioSource, USA) following the manufacturer's instructions. The optical density was measured at 450 nm.

For biochemical experiments, each sample was measured three times.

2.6. Survey of gene expression

RNA was extracted using a SinaClon kit (Iran). The quality of the extracted RNA was assessed at 260 nm, and the A260/A280 ratio was determined. Complementary DNA (cDNA) was synthesized with the Sinnaclon First Strand cDNA Synthesis Kit (SinaClon, Iran). Real-time PCR was conducted to evaluate gene expression. Each reaction mixture included 1 µl of primers, 2 µl of cDNA, 6 µl of double-distilled water, and 10 µl of qPCRBIO SyGreen Mix Lo-ROX. To standardize the expression levels of the target genes, we used the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as its expression remains stable in AD brains.²⁶ All experiments were performed in triplicate, and the relative expression of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method.²⁷ Primer 3 software was utilized to design and verify primers, using the NCBI BLAST for confirmation. The primer sequences are provided in Table 1.

2.7. Histological investigations

The samples were first fixed in 4% paraformaldehyde. After processing, they were embedded in paraffin, and 5-µm sections were prepared from the hippocampus, specifically from 2.7 mm to 3.7

mm posterior to bregma. Then, the sections were stained with 0.1% cresyl violet (Merck, Germany) for Nissl staining.²⁴

For thioflavin-S staining, sections were deparaffinized and hydrated. After that, they were incubated for 10 to 15 min at room temperature in 1% aqueous thioflavin-S (Sigma-Aldrich, USA). Then, the sections were washed three times with distilled water, glycerol, and PBS solution. Finally, a coverslip was placed on the sample for fluorescent photography.²⁸

Ten sections from the CA1 region of the hippocampus of each rat were examined microscopically after being stained with cresyl violet to assess neuronal death. Additionally, an equal number of sections stained with thioflavin were studied to determine the percentage of amyloid plaque deposition.

2.8. Statistical analysis

Data analysis was performed using SPSS version 26. A one-way analysis of variance (ANOVA) was conducted, followed by Tukey's post-hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. Results are expressed as mean $\pm$ standard error (SE).

3. Results

3.1. Characterization of nano AA

The image of nano AA, prepared using FESEM, is presented in Figure 1A. The image shows that the nanoparticles are nearly spherical and aggregated, with diameters of less than 0.2 μm . The

results from the DLS test further confirm this finding, reporting a diameter of 125 nm for the AA nanoparticles (Figure 1B).

3.2. Oxidative stress indicators

ICV injection of A β ₁₋₄₂ significantly reduced SOD activity in the hippocampus of the A β group compared to the Control group ($p < 0.001$). However, SOD activity was extremely high in both the A β .AA and A β .nAA groups compared to the A β group ($p < 0.001$). Moreover, the SOD activity in the A β .nAA group was significantly greater than that in the A β .AA group ($p < 0.001$) (Figure 2A).

Additionally, MDA levels were significantly elevated in the A β group compared to the Control group ($p < 0.001$). Both AA and nano AA treatments effectively prevented the rise in MDA levels; consequently, MDA levels in the A β .AA and A β .nAA groups were remarkably lower than the A β group ($p < 0.001$). Furthermore, MDA levels in the A β .nAA group were significantly lower than those in the A β .AA group ($p < 0.001$) (Figure 2B).

It is noteworthy that the A β .C group did not show significant differences from the A β group regarding any of these factors.

3.3. MCP-1 gene expression

The results of real-time PCR indicated that the expression of MCP-1 in the hippocampus of the A β group was significantly higher than in the Control group ($p < 0.001$). However, the expression of MCP-1 significantly decreased in both the A β .AA and A β .nAA groups compared to the A β group ($p < 0.001$). Additionally, the mRNA level of MCP-1 in the A β .nAA group was extremely

low than that in the A β .AA group ($p < 0.001$). There was no significant difference in MCP-1 expression between the A β and A β .C groups (Figure 3).

3.4. BACE-1 expression and amyloid plaque deposition

BACE-1 expression was significantly higher in the A β group compared to the Control group ($p < 0.001$). However, its expression decreased significantly in the A β .AA and A β .nAA groups compared to the A β group ($p < 0.01$ and $p < 0.001$, respectively). Additionally, the A β .nAA group exhibited a considerable decrease in BACE-1 expression compared to the A β .AA group ($p < 0.05$). There was no significant difference observed between the A β and A β .C groups (Figure 4A).

ICV injection of A β_{1-42} resulted in the deposition of amyloid plaques in the CA1 region of the hippocampus, as indicated by thioflavin staining shown in Figure 3B. The percentage of amyloid plaque deposition in the A β group was remarkably higher than in the Control group ($p < 0.001$). Both the AA and nano AA treatments were effective in reducing the amyloid deposition in A β_{1-42} -injected animals ($p < 0.001$). However, there was a significant difference between the A β .AA and A β .nAA groups ($p < 0.001$). The percentage of amyloid plaques in the A β .C group did not differ significantly from that in the A β group (Figure 4B, C).

3.5. Neuronal death

Microscopic examination of photomicrographs from Nissl-stained sections revealed a reduction in the thickness of the pyramidal neuron layer and significant neuronal death in the CA1 region of the hippocampus in the A β group. The percentage of viable neurons in the A β .AA and A β .nAA groups was significantly higher than in the A β group ($p < 0.001$). Furthermore, neuronal viability in the A β .nAA group was notably greater compared to the A β .AA group ($p < 0.001$) (Figure 5).

However, there was no significant difference in neuronal viability between the A β .C group and the A β group.

3.6. Tryptophan metabolism

IDO-1 expression in the hippocampus of A β group was significantly higher than that in the Control group ($p < 0.001$). However, it was significantly lower than the levels observed in the A β group when comparing the A β .AA and A β .nAA groups ($p < 0.001$). Additionally, there was a notable decrease in IDO-1 expression in the A β .nAA group compared to the A β .AA group ($p < 0.05$) (Figure 6A).

Injection of A β_{1-42} resulted in a significant decrease in serotonin levels and a considerable increase in kynurenine levels in the hippocampus of rats ($p < 0.001$). Serotonin levels in the A β .AA and A β .nAA groups were significantly higher than those in the A β group ($p < 0.001$). Conversely, the kynurenine levels in these groups were significantly lower than those in the A β group ($p < 0.001$). The A β .nAA group exhibited higher serotonin levels ($p < 0.01$) and lower kynurenine levels ($p < 0.001$) compared to the A β .AA group (Figure 6B).

The A β .C group did not show any meaningful differences from the A β group in any of these parameters (Figure 5).

3.7. Spatial learning and memory

In the MWM test, animals in the A β group spent significantly more time swimming to locate the hidden platform on the third and fourth days ($p < 0.01$ and $p < 0.001$, respectively). In contrast, the A β .AA and A β .nAA groups needed less time to reach the platform compared to the A β group on the fourth day ($p < 0.01$) (Figure 7A).

During the probe test, which was conducted without the platform, the A β group spent significantly less time in the target quadrant (the platform's location during training) compared to the Control group ($p < 0.001$). Conversely, both the A β .AA and A β .nAA groups spent more time in the target quadrant than the A β group ($p < 0.01$) (Figure 7B).

Throughout this portion of the study, there were no significant differences observed between the A β .AA and A β .nAA groups. Additionally, no substantial differences were found between the A β and A β .C groups (Figure 7).

4. Discussion

The results of the current study indicate that ICV injection of A β_{1-42} resulted in oxidative stress, neuroinflammation, the deposition of amyloid plaques, neuronal death, and altered tryptophan metabolism in the hippocampus of rats. Additionally, the learning and spatial memory abilities were impaired. These findings align with previous studies regarding the induction of AD by A β_{1-42} .^{29, 30}

The brain's vulnerability to oxidative stress plays a critical role in the development of AD, as there is a bidirectional relationship between A β and oxidative stress, where each factor exacerbates the other. Consequently, antioxidant therapy has been proposed as a potential therapeutic strategy for AD.³¹

AA serves not only as a free radical scavenger but also interacts with small-molecule antioxidants like tocopherol and glutathione. It stimulates the synthesis and activation of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase. Additionally, AA enhances the activity of

transcription factors, including nuclear factor erythroid 2-related factor 2 (Nrf2), which is crucial for the expression of genes that encode antioxidant proteins.³² AA does not cross the BBB; instead, the sodium vitamin C transporter 2 (SVCT2) is the primary transporter of AA in the brain, with high expression levels found in neurons.³³ The concentration of AA varies across different brain regions, with the highest levels identified in the hippocampus and hypothalamus.³⁴ As plasma AA levels decline, the brain is one of the last tissues to lose AA because astrocytes in the brain recycle it. However, an increase in ROS levels within the CNS impairs AA recycling, leading to increased oxidative damage in neurons.³³ Conversely, reduced expression of SVCT2 in AD model mice contributes to greater oxidative stress in the brain and increases the deposition of A β ₁₋₄₂ in the hippocampus and cortex.³⁵ In this study, a one-month treatment with AA and nano AA prevented the decrease in SOD activity and the rise in MDA production in the hippocampus of AD model rats, with nano AA proving to be more effective than AA in this regard. Given that chitosan-based nanomaterials allow for continuous drug release¹⁵, it is possible that chitosan nanoparticles carrying AA, which can cross the BBB through a mechanism independent of SVCT2, may help maintain AA levels in the brains of AD animal models by gradually releasing AA. It is important to emphasize that AA levels are crucial for its antioxidant function in the brain. For instance, when the hippocampus is exposed to ozone, AA reduces lipid peroxidation in a dose-dependent manner; however, its ability to mitigate lipid peroxidation diminishes at both low and very high doses.¹³ One limitation of this study is the lack of measurement of AA levels in the hippocampus of animals across the different treatment groups. Additionally, examining SVCT2 expression is recommended, as its levels may be altered in the A β -AA and A β .nAA groups.

Research on the pathobiology of various diseases has demonstrated a bidirectional relationship between oxidative stress and MCP-1.³⁶ MCP-1 has been identified in senile plaques (amyloid

deposits) within the brains of patients with AD.³⁷ It has been proposed that among inflammatory factors, MCP-1 is the most reliable predictor of AD.³⁸ Given the ability of AA and nano AA to reduce oxidative stress in the hippocampus of AD model rats, a decrease in MCP-1 expression can also be anticipated. Furthermore, previous studies have shown that AA can lower the expression of inflammatory factors such as MCP-1.³⁹

MCP-1 plays a significant role in the development of inflammation. Research indicates that MCP-1-deficient mice (MCP-1^{-/-}) exhibit reduced neuroinflammation following lipopolysaccharide injections compared to normal mice.⁴⁰ In AD, the interaction between astrocytes and microglia is crucial for the accumulation of A β and neuroinflammation. Astrocytes recruit microglia to areas of A β deposition by releasing chemokines such as MCP-1. Subsequently, astrocytes secrete cytokines that activate microglia, inducing a reactive state. Activated microglia also release cytokines, creating a feedback loop that exacerbates neuroinflammation. This bidirectional signaling can sustain a chronic inflammatory state that hinders A β clearance and promotes amyloid deposition.⁴¹ Moreover, increased inflammatory factors enhance the activity of beta-secretase and gamma-secretase in the hippocampus and prefrontal cortex.⁴² Microscopic examinations of thioflavin-stained hippocampal slices revealed that amyloid plaque deposition in AD model rats treated with AA and nano AA was significantly reduced. This reduction was anticipated due to decreased expression of MCP-1 and BACE-1, as well as a decline in oxidative stress in these animals. Supporting these findings, a study by Sampaio et al.¹⁰ demonstrated that there is competition between AA and A β ₁₋₄₂ monomers for binding to oligomers; notably, AA can destabilize small oligomers and may even reverse amyloid fibrillation. The present study indicated that nano AA was more effective than AA in reducing the expression of MCP-1 and BACE-1, thereby inhibiting the deposition of amyloid plaques.

One of the key events in AD is the widespread death of neurons in various brain regions, particularly the hippocampus. This neuronal death can occur through several mechanisms, including apoptosis, necroptosis, autophagy, pyroptosis, and ferroptosis. Several factors, including A β , hyperphosphorylated tau, oxidative stress, and inflammation, contribute to this process.⁴³ Based on *in vitro* and *in vivo* studies, MCP-1 can induce neurotoxicity by activating microglia. As a result, mice lacking MCP-1 exhibit greater resistance to neuronal death.⁴⁴ Therefore, both nano AA and AA, by reducing MCP-1 expression and preventing A β deposition in the hippocampus, also help protect pyramidal neurons from death. One study demonstrated that AA reduced A β -induced neuronal death in SH-SY5Y neuroblastoma cells and even decreased the basal generation of endogenous A β .⁴⁵ In another study involving rats fed a high-fat, high-fructose diet, AA chitosan nanoparticles, at a concentration one-tenth that of AA, demonstrated similar efficacy in protecting cortical and hippocampal neurons.⁴⁶ Similarly, in the present study, neuronal death was lower in the A β .nAA group compared to the A β .AA group.

Research has demonstrated that tryptophan metabolism may serve as a potential therapeutic candidate for AD. Certain metabolites of tryptophan function as neuroprotective agents, while others may contribute to the progression of AD. The primary metabolic pathway for tryptophan is the kynurenine pathway, in which the enzyme IDO-1 plays a crucial role. Patients with AD exhibit elevated kynurenine/tryptophan ratios, and IDO-1 activity is increased in the brains of animal models of AD. This heightened activity leads to neuroinflammation, A β accumulation, and neuronal death. Both neurons and glial cells appear to be sources of IDO-1, and they enhance IDO-1 activity by releasing inflammatory factors.^{47, 48} Also, Coptisine, an uncompetitive inhibitor of IDO-1, prevents neuronal loss, reduces the formation of amyloid plaques, and improves cognitive function in AD model mice.⁴⁹ Another pathway of tryptophan metabolism leads to the production

of serotonin. Studies indicate that higher levels of serotonin are linked to increased volume in the hippocampus and enhanced cognitive function in patients with AD.⁵⁰ In addition, the use of serotonin or even selective serotonin reuptake inhibitors has been found to decrease A β deposition in the brains of both AD patients and animal models.⁵¹ Consequently, the ability of AA and nano AA to lower IDO-1 expression, decrease kynurenine levels, and increase serotonin in the hippocampus of AD model rats played a critical role in reducing neuroinflammation, minimizing amyloid plaque deposition, and decreasing neuronal death in this brain region. Research has also indicated that AA has an antidepressant effect in mice by raising serotonin levels in the brain.⁵² Furthermore, AA chitosan nanoparticles are more effective than AA in enhancing serotonin turnover in the cortex and hippocampus⁴⁶, which is consistent with our findings.

Memory impairment due to synaptic dysfunction is one of the earliest symptoms observed in patients with AD. The accumulation of A β and hyperphosphorylation of tau proteins disrupts synaptic function, ultimately leading to synapse loss. Additionally, impairments in the neurotransmission of acetylcholine, glutamate, serotonin, dopamine, and noradrenaline, along with the disruption of proteins involved in synaptic structure and function, contribute to memory deficits in these patients.^{2, 53} In the present study, spatial learning and memory in the A β -AA and A β -nAA groups showed significant improvement compared to the A β group. One study indicated that the memory improvement induced by AA in an AD model of mice occurs through the reduction of A β oligomerization, a significant decrease in brain oxidative damage, increased expression of synaptophysin, and decreased phosphorylation of tau at Ser396.⁵⁴ In our study, while nano AA was more effective than AA in preventing A β deposition and increasing serotonin levels, its ability to enhance memory was similar to that of AA. Since various neurotransmitters are involved in memory formation, it is necessary to consider their contributions. The lack of

examination of these neurotransmitter levels in the hippocampus across different groups can be viewed as a limitation of this study.

A β oligomers inhibit the long-term potentiation of glutamate-dependent N-methyl-D-aspartate (NMDA) receptors.⁵⁵ A β also increases the expression of the NMDA receptor subunit 2B (NR2B) in the hippocampus, which is associated with enhanced calcium release and reduced neuronal survival.⁵⁶ Among acetylcholine receptors, the alpha-7-nicotinic acetylcholine receptor (α 7-nAChR), which is abundantly expressed in the hippocampus and cortex, is affected in AD. Therefore, agonists of this receptor have been considered for their potential to reduce A β production and improve cognitive functions in AD.⁵⁷ In a study conducted by Kara et al.⁵⁸, ascorbic acid treatment (250 mg/kg) for 10 weeks did not impact the expression of α 7-nAChR or NR2B in the hippocampus of rats, nor did it alter spatial memory. However, it is important to note that the expression of these receptors is altered in AD, as previously mentioned. One limitation of this study is the lack of examination of the expression and activity of cholinergic and glutamatergic receptors across different experimental groups.

Current mechanistic approaches to developing AD therapies using nanoparticles focus on several key strategies. These include the clearance of A β fibrils and aggregates, the creation of nanoparticles loaded with acetylcholinesterase inhibitors to improve cholinergic system function, the reduction of neuroinflammation, the inhibition of tau hyperphosphorylation, and the development of anti-A β peptide antibodies delivered via circulating nanoparticles. This last strategy initiates a "sink mechanism," effectively removing soluble A β peptides from the brain into the bloodstream.⁵⁹ In the present study, AA loaded in chitosan nanoparticles demonstrated greater effectiveness in reversing the molecular, biochemical, and histological changes caused by

A β in the hippocampus compared to AA alone, although both produced similar behavioral outcomes. These positive effects are likely attributed to the increased levels of AA in the brain, facilitated by the ability of chitosan nanoparticles to pass easily through the BBB. Future studies should explore the impact of nano AA on other therapeutic targets, such as tau, and investigate different dosages of this nanoparticle over both shorter and longer periods.

In summary, incorporating ascorbic acid into chitosan nanoparticles boosts the vitamin's effectiveness in reducing oxidative stress, lowering neuroinflammation, and preventing neuronal death. This approach also aids in regulating tryptophan metabolism and decreases the deposition of amyloid plaques in a rat model of AD. However, it does not produce any changes in memory improvement.

Authors' Contribution

MP: Investigation, Visualization. MA-R: Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. MS and AE: Conceptualization, Methodology.

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Conflicts of Interest

The authors declare that they have no competing interests.

Data Availability Statement

Data will be available upon request from the corresponding authors.

References

1. Chen Y, Fu AK, Ip NY. Synaptic dysfunction in Alzheimer's disease: Mechanisms and therapeutic strategies. *Pharmacol Ther.* 2019;195:186-198. doi: 10.1016/j.pharmthera.2018.11.006
2. Long JM, Holtzman DM. Alzheimer disease: an update on pathobiology and treatment strategies. *Cell.* 2019;179(2):312-339. doi: 10.1016/j.cell.2019.09.001
3. Ju Y, Tam KY. Pathological mechanisms and therapeutic strategies for Alzheimer's disease. *Neural Regen Res.* 2022;17(3):543-549. doi: 10.4103/1673-5374.320970
4. Fanlo-Ucar H, Picón-Pagès P, Herrera-Fernández V, Ill-Raga G, Muñoz FJ. The Dual role of amyloid beta-peptide in oxidative stress and inflammation: unveiling their connections in Alzheimer's disease etiopathology. *Antioxidants.* 2024;13(10):1208. doi: 10.3390/antiox13101208
5. Pardo-Moreno T, González-Acedo A, Rivas-Domínguez A, García-Morales V, García-Cozar FJ, Ramos-Rodríguez JJ, et al. Therapeutic approach to Alzheimer's disease: current treatments and new perspectives. *Pharmaceutics.* 2022;14(6):1117. doi: 10.3390/pharmaceutics14061117
6. Pritam P, Deka R, Bhardwaj A, Srivastava R, Kumar D, Jha AK, et al. Antioxidants in Alzheimer's disease: current therapeutic significance and future prospects. *Biology.* 2022;11(2):212. doi: 10.3390/biology11020212
7. Njus D, Kelley PM, Tu YJ, Schlegel HB. Ascorbic acid: The chemistry underlying its antioxidant properties. *Free Radic Biol Med.* 2020;159:37-43. doi: 10.1016/j.freeradbiomed.2020.07.013

8. Gęgotek A, Skrzydlewska E. Antioxidative and anti-inflammatory activity of ascorbic acid. *Antioxidants*. 2022;11(10):1993. doi: 10.3390/antiox11101993
9. Hamid M, Mansoor S, Amber S, Zahid S. A quantitative meta-analysis of vitamin C in the pathophysiology of Alzheimer's disease. *Front Aging Neurosci*. 2022;14:970263. doi: 10.3389/fnagi.2022.970263
10. Sampaio I, Quatroni FD, Lins PM, Nascimento AS, Zucolotto V. Modulation of beta-amyloid aggregation using ascorbic acid. *Biochimie*. 2022;200:36-43. doi: 10.1016/j.biochi.2022.05.006
11. Kook SY, Lee KM, Kim Y, Cha MY, Kang S, Baik SH, et al. High-dose of vitamin C supplementation reduces amyloid plaque burden and ameliorates pathological changes in the brain of 5XFAD mice. *Cell Death Dis*. 2014;5(2):e1083. doi: 10.1038/cddis.2014.26
12. Olajide OJ, Yawson EO, Gbadamosi IT, Arogundade TT, Lambe E, Obasi K, et al. Ascorbic acid ameliorates behavioural deficits and neuropathological alterations in rat model of Alzheimer's disease. *Environ Toxicol Pharmacol*. 2017;50:200-211. doi: 10.1016/j.etap.2017.02.010
13. Figueroa-Méndez R, Rivas-Arancibia S. Vitamin C in health and disease: its role in the metabolism of cells and redox state in the brain. *Front Physiol*. 2015;6:397. doi: 10.3389/fphys.2015.00397
14. Cao Y, Zhang R. The application of nanotechnology in treatment of Alzheimer's disease. *Front Bioeng Biotechnol*. 2022;10:1042986. doi: 10.3389/fbioe.2022.1042986
15. Patra JK, Das G, Fraceto LF, Campos EV, Rodriguez-Torres MD, Acosta-Torres LS, et al. Nano based drug delivery systems: recent developments and future prospects. *J nanobiotechnology*. 2018;16:71. doi: 10.1186/s12951-018-0392-8

16. Manek E, Darvas F, Petroianu GA. Use of biodegradable, chitosan-based nanoparticles in the treatment of Alzheimer's disease. *Molecules*. 2020;25(20):4866. doi: 10.3390/molecules25204866
17. Gan Q, Wang T, Cochrane C, McCarron P. Modulation of surface charge, particle size and morphological properties of chitosan–TPP nanoparticles intended for gene delivery. *Colloids Surf B Biointerfaces*. 2005;44(2-3):65-73. doi: 10.1016/j.colsurfb.2005.06.001
18. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *J Cereb Blood Flow Metab*. 2020;40(9):1769-1777. doi: 10.1177/0271678X20943823
19. Karakelle NA, Dinçer S, Sağlam AS. The effect of intracerebroventricular amyloid beta 1–42 application on cognitive functions in aged rats supplemented with taurine and the change of peroxisomal proteins in this process. *Brain Res Bull* 2021;172:89-97. doi: 10.1016/j.brainresbull.2021.04.011
20. Bilginaylar K, Aykac A, Sayiner S, Özkayalar H, Şehirli AÖ. Evaluation of the antiapoptotic and anti-inflammatory properties of chitosan in methotrexate-induced oral mucositis in rats. *Mol Biol Rep*. 2022;49(4):3237-3245. doi: 10.1007/s11033-022-07158-x
21. Rauf N, Nawaz A, Ullah H, Ullah R, Nabi G, Ullah A, et al. Therapeutic effects of chitosan-embedded vitamin C, E nanoparticles against cisplatin-induced gametogenic and androgenic toxicity in adult male rats. *Environ Sci Pollut Res*. 2021;28(40):56319-56332. doi: 10.1007/s11356-021-14516-y
22. Oh SS, Narver HL. Mouse and rat anesthesia and analgesia. *Curr Protoc*. 2024;4(2):e995. doi: 10.1002/cpz1.995

23. Paxinos G, Watson C. The rat brain in stereotaxic coordinates, 2006, 6th ed. Academic press, New York.
24. Asle-Rousta M, Peirovy Y. Neuroprotective effects of thymol and p-cymene in immobilized male rats through alterations in molecular, biochemical, histological, and behavioral parameters. *Neurochem Res.* 2025;50(1):5. doi: 10.1007/s11064-024-04271-0
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265–275. doi: 10.1016/S0021-9258(19)52451-6
26. Gutala RV, Reddy PH. The use of real-time PCR analysis in a gene expression study of Alzheimer's disease post-mortem brains. *J Neurosci Methods.* 2004;132:101-107. doi: 10.1016/j.jneumeth.2003.09.005
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001;25:402-408. doi: 10.1006/meth.2001.1262
28. Radfar F, Shahbazi M, Tahmasebi Boroujeni S, Arab Ameri E, Farahmandfar M. Moderate aerobic training enhances the effectiveness of insulin therapy through hypothalamic IGF1 signaling in rat model of Alzheimer's disease. *Sci Rep* 2024;14:15996. doi: 10.1038/s41598-024-66637-2
29. Min LJ, Kobayashi Y, Mogi M, Tsukuda K, Yamada A, Yamauchi K, et al. Administration of bovine casein-derived peptide prevents cognitive decline in Alzheimer disease model mice. *PloS one.* 2017;12(2):e0171515. doi: 10.1371/journal.pone.0171515

30. Basir HS, Mirazi N, Komaki A, Mohamadpour B, Hosseini A. Selegiline improves cognitive impairment in the rat model of Alzheimer's disease. *Mol Neurobiol*. 2025;62(2):2548-2560. doi: 10.1007/s12035-024-04388-x
31. Tamagno E, Guglielmotto M, Vasciaveo V, Tabaton M. Oxidative stress and beta amyloid in Alzheimer's disease. Which comes first: the chicken or the egg?. *Antioxidants*. 2021;10(9):1479. doi: 10.3390/antiox10091479
32. Gęgotek A, Skrzydlewska E. Ascorbic acid as antioxidant. *Vitam Horm*. 2023;121:247-270. doi: 10.1016/bs.vh.2022.10.008
33. Nualart F, Mack L, García A, Cisternas P, Bongarzone ER, Heitzer M, et al. Vitamin C transporters, recycling and the bystander effect in the nervous system: SVCT2 versus gluts. *Stem Cell Res Ther*. 2014;4(5):209. doi: 10.4172/2157-7633.1000209
34. Oke AF, May L, Adams RN. Ascorbic acid distribution patterns in human brain: A comparison with nonhuman mammalian species. *Ann N Y Acad Sci*. 1987;498(1):1-12. doi: 10.1111/j.1749-6632.1987.tb23747.x
35. Dixit S, Bernardo A, Walker JM, Kennard JA, Kim GY, Kessler ES, et al. Vitamin C deficiency in the brain impairs cognition, increases amyloid accumulation and deposition, and oxidative stress in APP/PSEN1 and normally aging mice. *ACS Chem Neurosci*. 2015;6(4):570-581. doi: 10.1021/cn500308h
36. Singh S, Anshita D, Ravichandiran V. MCP-1: Function, regulation, and involvement in disease. *Int Immunopharmacol*. 2021;101:107598 doi: 10.1016/j.intimp.2021.107598

37. Ishizuka K, Kimura T, Igata-yi RU, Katsuragi S, Takamatsu J, Miyakawa T. Identification of monocyte chemoattractant protein-1 in senile plaques and reactive microglia of Alzheimer's disease. *Psychiatry Clin Neurosci*. 1997;51(3):135-138. doi: 10.1111/j.1440-1819.1997.tb02375.x
38. Sokolova A, Hill MD, Rahimi F, Warden LA, Halliday GM, Shepherd CE. Monocyte chemoattractant protein-1 plays a dominant role in the chronic inflammation observed in Alzheimer's disease. *Brain Pathol*. 2009;19(3):392-398. doi: 10.1111/j.1750-3639.2008.00188.x
39. Majewicz J, Rimbach G, Proteggente AR, Lodge JK, Kraemer K, Minihane AM. Dietary vitamin C down-regulates inflammatory gene expression in apoE4 smokers. *Biochem Biophys Res Commun*. 2005; 338(2):951-955. doi: 10.1016/j.bbrc.2005.10.029
40. Thompson WL, Karpus WJ, Van Eldik LJ. MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. *J Neuroinflammation*. 2008;5:35. doi: 10.1186/1742-2094-5-35
41. Azargoonjahromi A. The duality of amyloid- β : its role in normal and Alzheimer's disease states. *Mol Brain*. 2024;17(1):44. doi: 10.1186/s13041-024-01118-1
42. Michelon C, Michels M, Abatti M, Vieira A, Borges H, Dominguni D, et al. The role of secretase pathway in long-term brain inflammation and cognitive impairment in an animal model of severe sepsis. *Mol Neurobiol*. 2020;57(2):1159-1169. doi: 10.1007/s12035-019-01808-1
43. Goel P, Chakrabarti S, Goel K, Bhutani K, Chopra T, Bali S. Neuronal cell death mechanisms in Alzheimer's disease: An insight. *Front Mol Neurosci*. 2022;15:937133. doi: 10.3389/fnmol.2022.937133

44. Yang G, Meng Y, Li W, Yong Y, Fan Z, Ding H, et al. Neuronal MCP-1 mediates microglia recruitment and neurodegeneration induced by the mild impairment of oxidative metabolism. *Brain Pathol.* 2011;21(3):279-297. doi: 10.1111/j.1750-3639.2010.00445.x
45. Huang J, May JM. Ascorbic acid protects SH-SY5Y neuroblastoma cells from apoptosis and death induced by β -amyloid. *Brain Res.* 2006;1097(1):52-58. doi: 10.1016/j.brainres.2006.04.047
46. Abd-Allah H, Nasr M, Ahmed-Farid OA, El-Marasy SA, Bakeer RM, Ahmed RF. Biological and pharmacological characterization of ascorbic acid and nicotinamide chitosan nanoparticles against insulin-resistance-induced cognitive defects: a comparative study. *ACS omega.* 2021;6(5):3587-3601. doi: 10.1021/acsomega.0c05096
47. Widner B, Leblhuber F, Walli J, Tilz GP, Demel U, Fuchs D. Tryptophan degradation and immune activation in Alzheimer's disease. *J Neural transm.* 2000;107:343-353. doi: 10.1007/s007020050029
48. Xie L, Wu Q, Li K, Khan MA, Zhang A, Sinha B, et al. Tryptophan metabolism in Alzheimer's disease with the involvement of microglia and astrocyte crosstalk and gut-brain axis. *Aging Dis.* 2024;15(5):2168. doi: 10.14336/AD.2024.0134
49. Yu D, Tao BB, Yang YY, Du LS, Yang SS, He XJ, et al. The IDO inhibitor coptisine ameliorates cognitive impairment in a mouse model of Alzheimer's disease. *J Alzheimer's Dis.* 2014;43(1):291-302. doi: 10.3233/JAD-14041
50. Azargoonjahromi A. Alzheimer's Disease Neuroimaging Initiative. Serotonin enhances neurogenesis biomarkers, hippocampal volumes, and cognitive functions in Alzheimer's disease. *Mol Brain.* 2024;17(1):93. doi: 10.1186/s13041-024-01169-4

51. Cirrito JR, Disabato BM, Restivo JL, Verges DK, Goebel WD, Sathyan A, et al. Serotonin signaling is associated with lower amyloid- β levels and plaques in transgenic mice and humans. *P Natl Acad Sci*. 2011;108(36):14968-14973. doi: 10.1073/pnas.1107411108
52. Maratha S, Sharma V, Walia V. Antidepressant like effect of ascorbic acid in mice: possible involvement of NO-sGC-cGMP signaling. *Neurochem Res*. 2022;47(4):967-978. doi: 10.1007/s11064-021-03496-7
53. Kamatham PT, Shukla R, Khatri DK, Vora LK. Pathogenesis, diagnostics, and therapeutics for Alzheimer's disease: Breaking the memory barrier. *Ageing Res Rev*. 2024;101:102481. doi: 10.1016/j.arr.2024.102481
54. Murakami K, Murata N, Ozawa Y, Kinoshita N, Irie K, Shirasawa T, et al. Vitamin C restores behavioral deficits and amyloid- β oligomerization without affecting plaque formation in a mouse model of Alzheimer's disease. *J Alzheimer's Dis*. 2011;26(1):7-18 doi: 10.3233/JAD-2011-101971
55. Xia W. Brain amyloid β protein and memory disruption in Alzheimer's disease. *Neuropsychiatr Dis Treat*. 2010:605-611. doi: 10.2147/NDT.S7460
56. Raïch I, Lillo J, Rebassa JB, Capó T, Cordoní A, Reyes-Resina I, et al. Dual role of NMDAR containing NR2A and NR2B subunits in Alzheimer's disease. *Int J Mol Sci*. 2024;25(9):4757. doi: 10.3390/ijms25094757
57. Singh S, Agrawal N, Goyal A. Role of Alpha-7-nicotinic acetylcholine receptor in Alzheimer's disease. *CNS Neurol Disord Drug Target*. 2024;23(3):384-394. doi: 10.2174/1871527322666230627123426

58. Kara Y, Doguc DK, Kulac E, Gultekin F. Acetylsalicylic acid and ascorbic acid combination improves cognition; Via antioxidant effect or increased expression of NMDARs and nAChRs?. *Environ Toxicol Pharmacol.* 2014;37(3):916-927. doi: 10.1016/j.etap.2014.02.019
59. Ribarič S. Nanotechnology Therapy for Alzheimer' s Disease Memory Impairment Attenuation. *Int J Mol Sci.* 2021;22(3):1102. doi: 10.3390/ijms22031102

Table 1. Primer sequences for real-time PCR.

Gene	Forward Primers Sequence (5'–3')	Reverse Primers Sequence (5'–3')
MCP-1	CTCACCTGCTGCTACTCATTCA	GACCCATTCCTTATTGGGGTCAG
BACE1	ATTTCTCCTGCCCCACTCTC	ATTCCCGAGCCCACCCATA
IDO1	GCATCAAGACCCGAAAGCAC	CACGAAGTCACGCATCCTC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Figure legends

Figure 1. (A) FESEM image of AA loaded chitosan nanoparticles. The red square represents the nanoparticle. (B) Average size distribution of nanoparticles obtained by DLS.

Figure 2. (A) SOD activity, and (B) MDA levels in the hippocampus. Values are presented as means $\pm$ SE from 5 animals per group (analyzed using one-way ANOVA and Tukey's post hoc test). *** $p < 0.001$ versus the Control group; #### $p < 0.001$ versus the A β group; and &&& $p < 0.001$ versus the A β .AA group.

Figure 3. MCP-1 expression in the hippocampus. Values are shown as means $\pm$ SE from 5 animals per group (analyzed using one-way ANOVA and Tukey's post hoc test). *** $p < 0.001$ versus the Control group; #### $p < 0.001$ versus the A β group; and &&& $p < 0.001$ versus the A β .AA group.

Figure 4. (A) BACE-1 expression, (B) photomicrographs of sections from the CA1 region of the hippocampus stained with thioflavin, and (C) comparison of the percentage of amyloid plaque in the CA1 region across different groups. Yellow arrows indicate amyloid plaques. Values are expressed as means $\pm$ SE from 5 animals per group (analyzed using one-way ANOVA and Tukey's post hoc test). *** $p < 0.001$ versus the Control group; ## $p < 0.01$, ### $p < 0.001$ versus the A β group; and & $p < 0.05$, && $p < 0.001$ versus the A β .AA group.

Figure 5. (A) Photomicrographs of sections from the CA1 region of the hippocampus stained with cresyl violet, and (B) comparison of the percentage of viable neurons in the CA1 region across different groups. Black arrows indicate a decrease in the thickness of the neuronal layer. *** $p < 0.001$ versus the Control group; ### $p < 0.001$ versus the A β group; and &&& $p < 0.001$ versus the A β .AA group.

Figure 6. (A) IDO-1 expression, and (B) levels of serotonin and kynurenine in the hippocampus. Values are expressed as means $\pm$ SE from 5 animals per group (analyzed using one-way ANOVA and Tukey's post hoc test). *** $p < 0.001$ versus the Control group; ### $p < 0.001$ versus the A β group; and & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ versus the A β .AA group.

Figure 7. (A) Mean time spent reaching the hidden platform during training days, and (B) time spent in the target quadrant during the probe test in the Morris Water Maze. Values are expressed as means $\pm$ SE from 10 animals per group (analyzed using one-way ANOVA and Tukey's post hoc test). ** $p < 0.01$ and *** $p < 0.001$ versus the Control group; ## $p < 0.01$ versus the A β group.

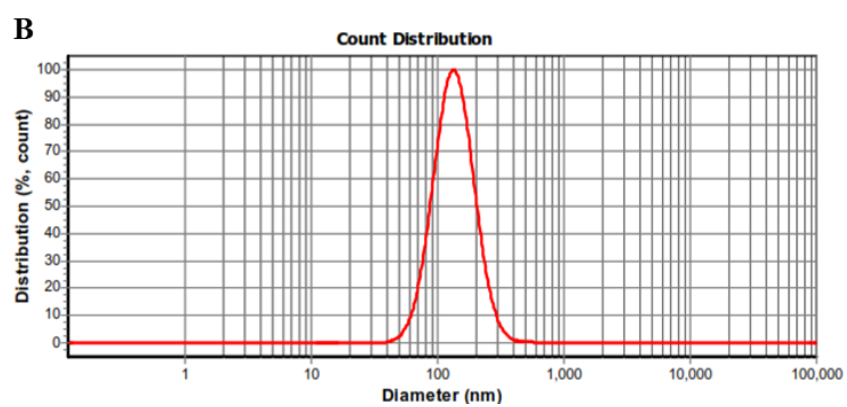
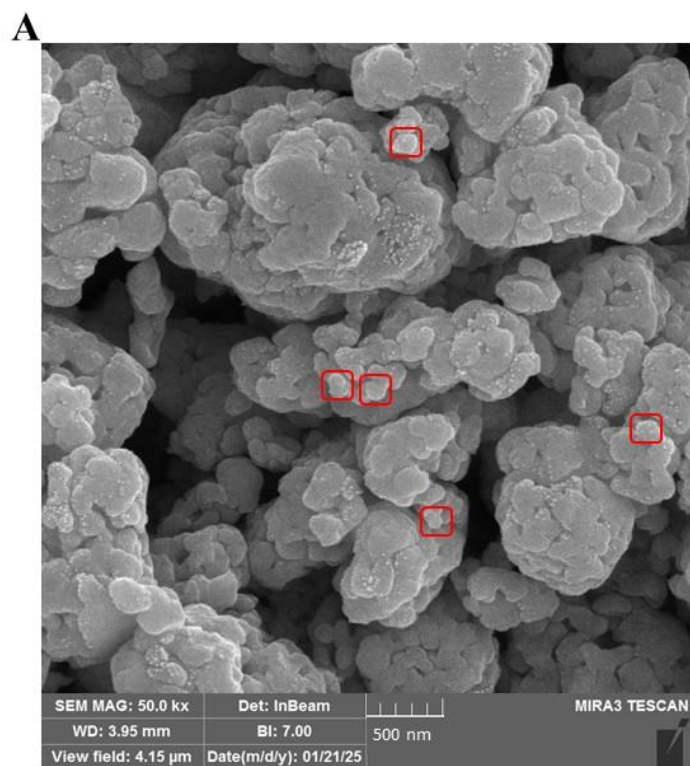


Figure 1.

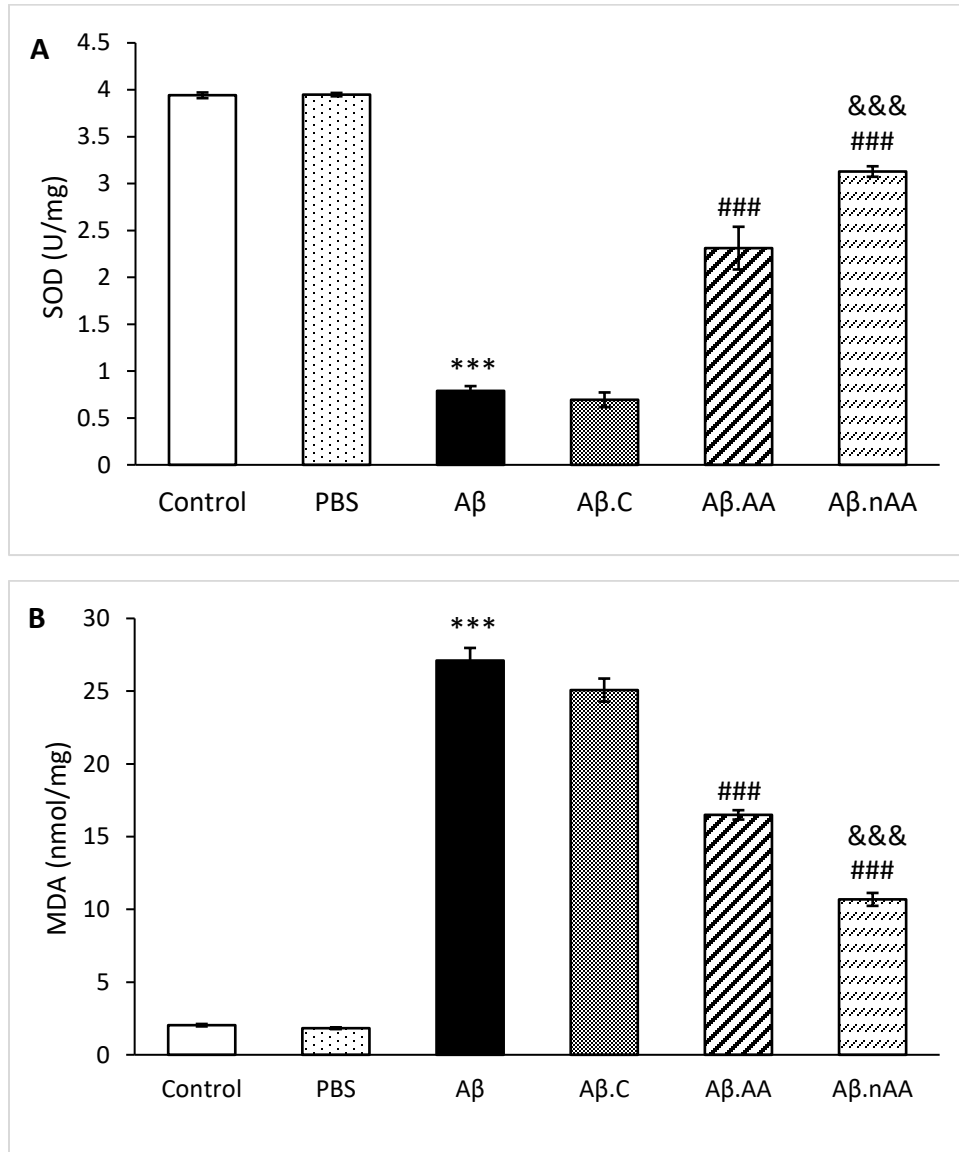


Figure 2.

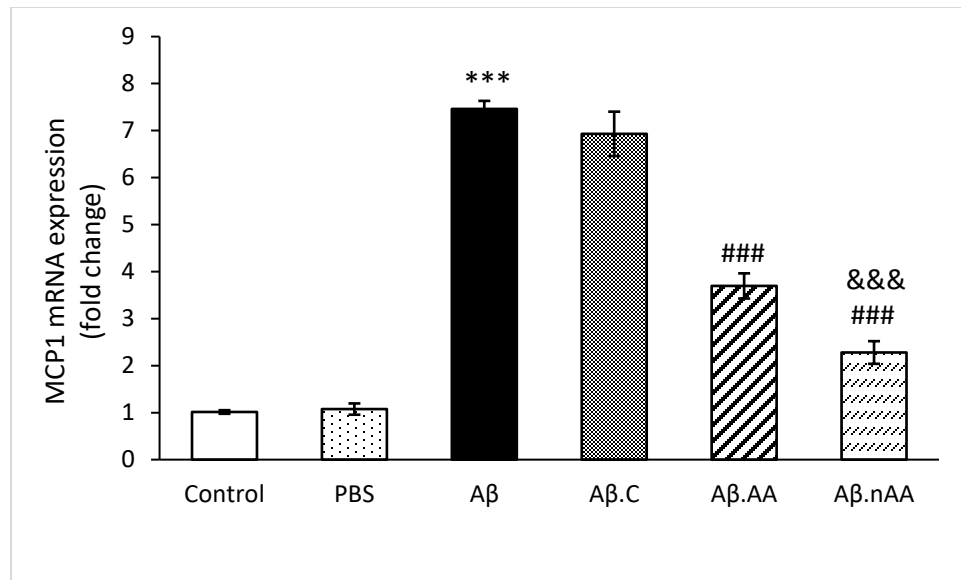
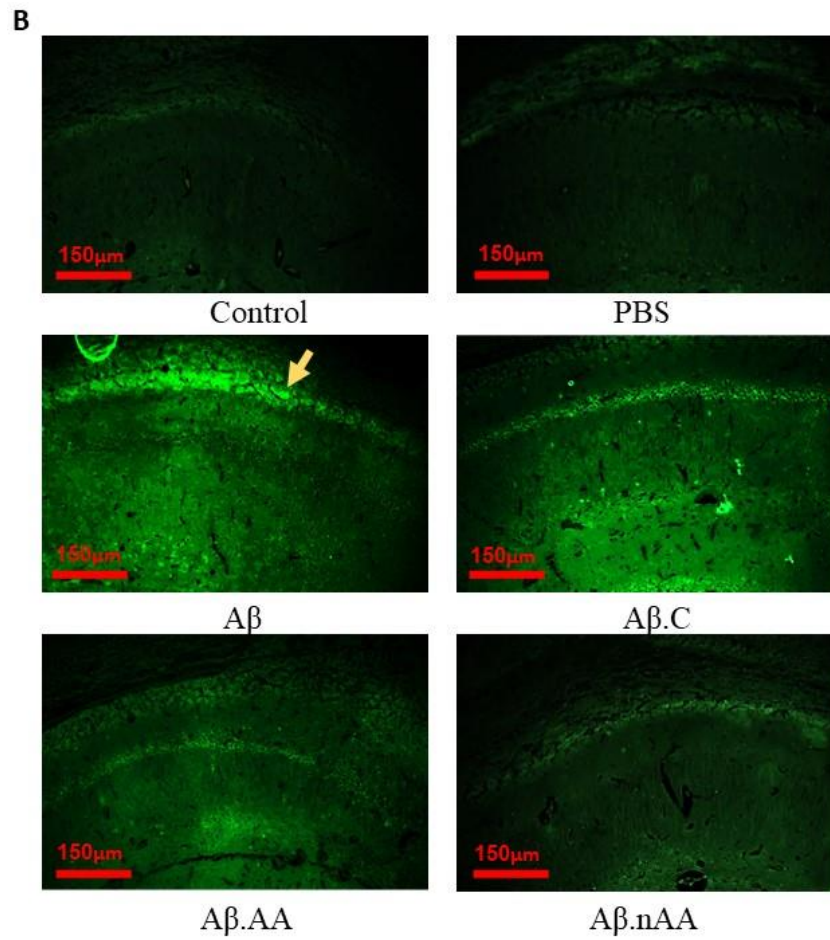
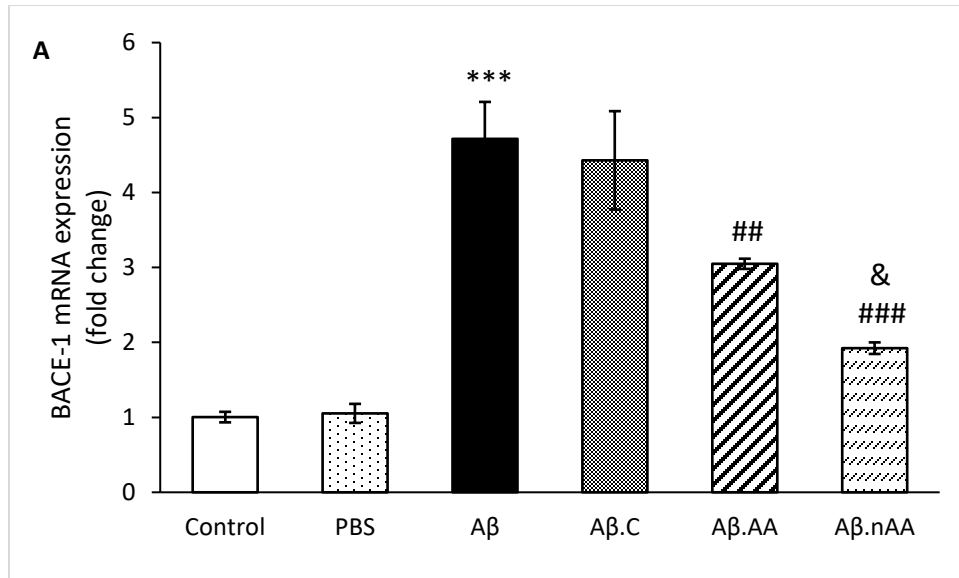


Figure 3.



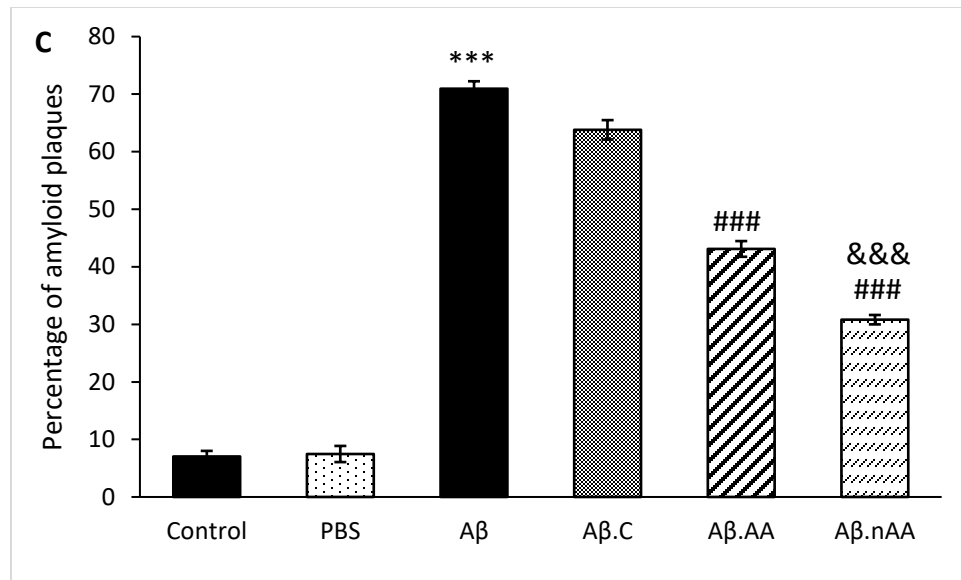


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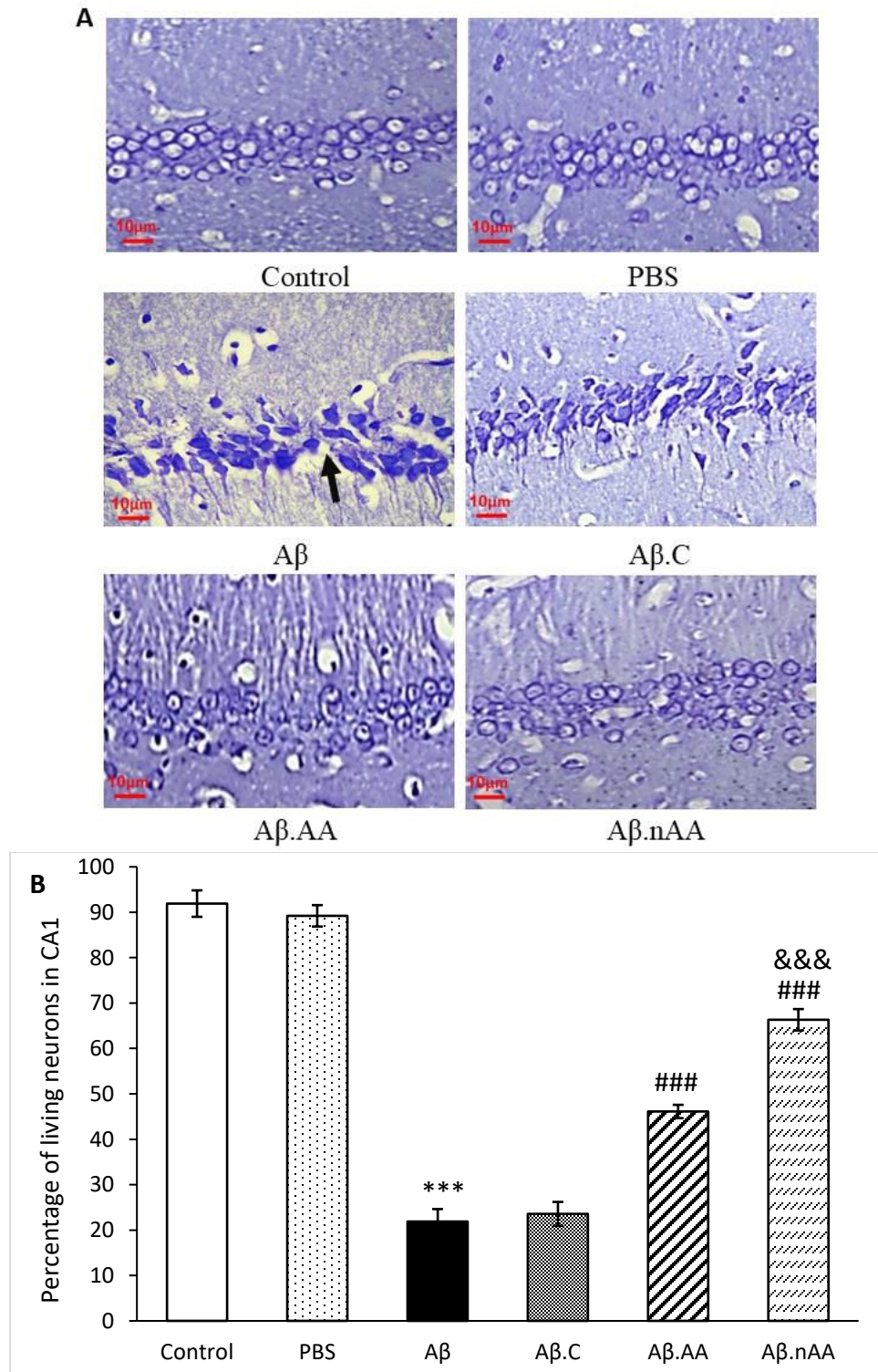


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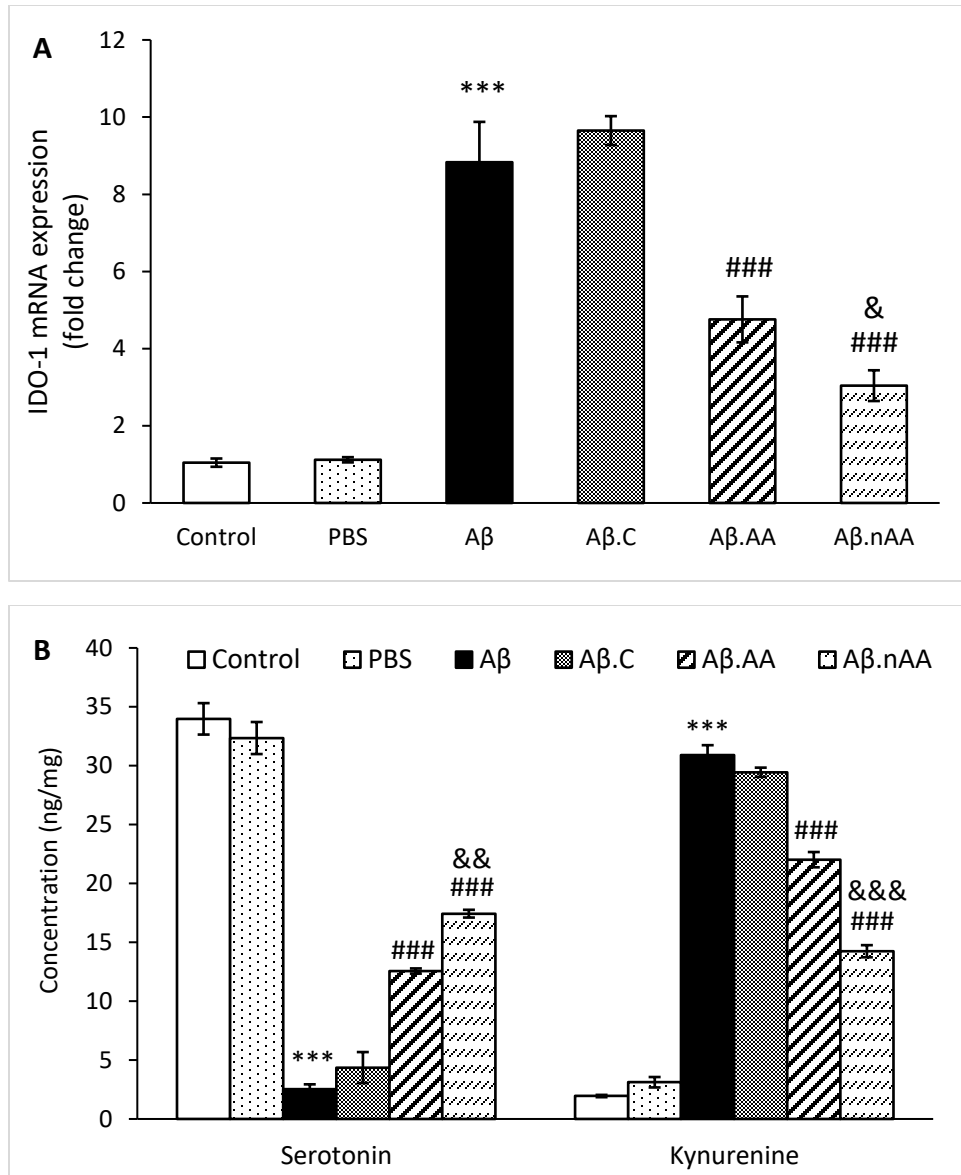


Figure 6.

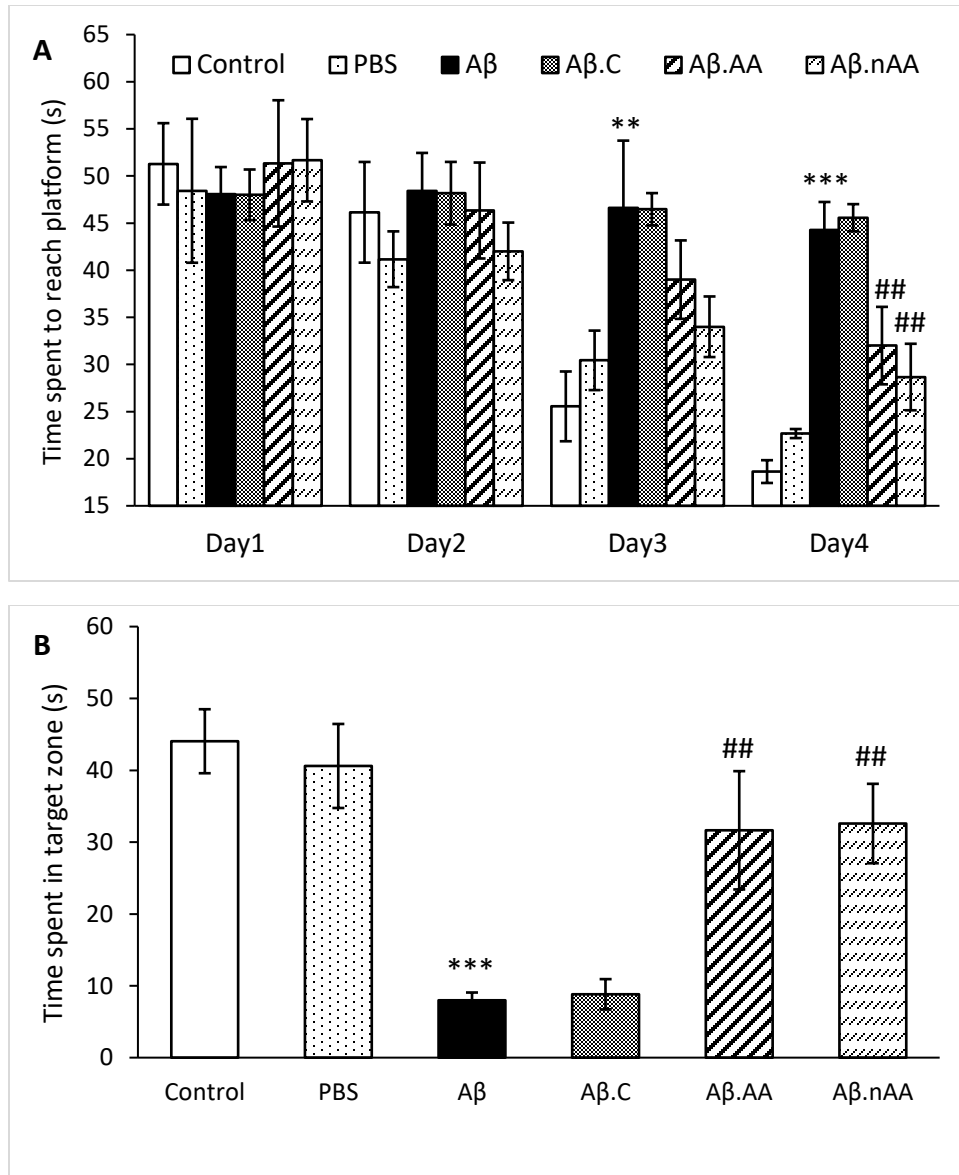


Figure 7.