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Donepezil-loaded PLGA-b-PEG Nanoparticles Enhance the Learning and Memory Function of Beta-Amyloid Rat Model of Alzheimer's Disease

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ABSTRACT

Introduction: Our aim is to reduce the side effects and increase the efficiency of donepezil by formulating donepezil-loaded poly(lactic-co-glycolic acid)-block-poly(ethylene glycol) nanoparticles (NPs) directly targeting amyloid beta (Aβ) fibrils in the brain and evaluate behavioral changes in this fibril model of AD.

Methods: AD model was developed by intracerebroventricular injection of pre-aggregated β25-35 fibrils. Rats were intravenously administered either solvent, donepezil-loaded NPs (15μg/kg) or free donepezil (1mg/kg) 3 times for a week except for naïve controls. The effect of treatments on anxiety, motor functions, and cognitive functions was tested by elevated plus maze, locomotor activity, novel object recognition, and Morris's water maze tests, respectively.

Results: Accumulation of Aβ25-35 fibrils in brain sections was confirmed. Anxiety-like behavior was observed in the Aβ Alzheimer and free donepezil treatment groups while donepezil-loaded NP treatment showed hypo-anxiety-like behavior. Donepezil-loaded NPs were successful in treatment of short-term memory deficit better than free donepezil injection. In Morris's water maze, both donepezil-loaded NPs and free donepezil groups found the platform in shorter time compared to Aβ Alzheimer group. In locomotor activity test, both donepezil treated groups moved less than the Aβ Alzheimer group and naïve controls. After the pharmacological experiments, acetylcholinesterase activity was determined and showed an increase in Aβ Alzheimer group compared to controls. Donepezil-loaded NPs inhibited the acetylcholinesterase activity more efficiently than the free donepezil group.

Conclusion: Targeting with donepezil-loaded PLGA-b-PEG-NPs increases efficiency, helps to inhibit acetylcholinesterase activity more substantially, improves cognitive decline due to its longer duration of action and destabilizing effect on amyloid fibrils.

Keywords: Alzheimer's disease, animal model, behavioral test, brain targeting, donepezil, nanoparticle

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INTRODUCTION

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease caused by neuronal loss in the hippocampus and cortex (1). Although the underlying pathology remains unclear, the deposition of the amyloid beta (Aβ) proteins in the extracellular synapses of the neocortex is suggested to play a major role in the progression of the disease (1,2). There is a decrease in choline acetyltransferase (ChAT) activity of frontal and parietal cortex, temporal lobe, and Meynert basal nuclei in AD (2).

Amyloid cascade and the cholinergic hypothesis are two major hypotheses suggested for the molecular mechanism of AD pathology. According to the amyloid cascade hypothesis, it is suggested that Aβ fibrils or phosphorylated tau accumulate and produce senile plaques and neurofibrillary tangles, which are toxic to the neurons and lead to synaptic loss and cell death (2–4). On the other hand, studies on animal models or brain samples of AD patients showed a significant decrease in cholinergic transmission, which causes cognitive symptoms such as memory impairment and behavioral changes (3–5).

AD is multifactorial, complex neurodegenerative disorder causing symptoms varying from oxidative stress to vascular dysfunction. Thus, creating an animal model that recapitulates the disease pathology and behavioral changes is essential. Based on the evidence, accumulation of Aβ is a key factor, therefore, animal models were created to target these histopathologic changes (3,6). Intracerebral Aβ injection is one of well-studied, characterized, successful, and practical methods to mimic AD and its cognitive and behavioral changes (6,7). Depending on structure-activity study, 25–35 areas of Aβ are responsible for stabile aggregation, thus, stereotaxic injection into rat brain leads to neuronal death eventually (7).

Important problems such as targeting drugs to a specific organ or tissue, extending the effective period of drugs, crossing the blood-brain barrier (BBB), localization of the drug, and developing nano systems for reducing side effects of the drugs have been studied for years with the aim of finding a solution (8). Hydrophilic and hydrophobic drugs, proteins, vaccines,

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Highlights

- **Donepezil-loaded PLGA-b-PEG Nanoparticles directed to the Aβ in brain.**
- **Donepezil-loaded PLGA-b-PEG NPs were successful in treatment of short-term memory deficit.**
- **Donepezil-loaded PLGA-b-PEG NPs improve the spatial learning and memory.**
- **Donepezil-loaded PLGA-b-PEG NPs inhibited the acetylcholinesterase activity more efficiently.**

and biological or macromolecules can be encapsulated and transported effectively with nanosystems (9,10). Thus, drugs can be targeted to many tissues, including the brain, and remain in the bloodstream at a sufficient concentration for the desired period (10).

AD still does not have a radical treatment but cholinesterase inhibitors, such as donepezil, are commonly used drugs for symptomatic treatments. Donepezil is a long-term inhibitor of cholinesterase in the brain and has the ability to inhibit Aβ fibril formation and protect against neuroinflammation (11). However, the main problem with the oral administration of donepezil is the gastrointestinal side effects like nausea, vomiting, and diarrhea. The short half-life and quick metabolization of the drug necessitate repetitive administration, which cause compliance problems, especially for patients suffering from dementia. Therefore, targeting donepezil to the brain might lessen the side effects, prolong the effective period and help patients with compliance problems.

The aim of the study is to evaluate the effect of donepezil-loaded PLGA-b-PEG nanoparticles (NPs) treatments on behavioral and cognitive functions in an AD animal model developed by intracerebroventricular (ICV) $A\beta_{25-35}$ peptide injection.

METHODS

Preparation of Donepezil-loaded PLGA-b-PEG Nanoparticles

Donepezil-loaded PLGA-b-PEG nanoparticles were prepared using water-in-oil-in-water (W/O/W) double emulsion method (12). Briefly, primary emulsion was created by homogenizing aqueous donepezil solution in PLGA-b-PEG–COOH polymer solution at 1,500 rpm for 1 min. Primary solution was immediately added dropwise into a secondary aqueous phase containing 2% Pluronic F68 and was homogenized at 15,000 rpm for 1 min. This second emulsion was added dropwise into 0.5% Pluronic F68 solution while stirring on a magnetic stirrer. The obtained emulsion was stirred overnight at 4˚C to remove residual solvent. Formed nanoparticles were centrifuged at 20,000x g for 20 min. After discarding the supernatants, nanoparticles were washed using centrifugation 3 times at 20,000x g. Destabilizing effect of these donepezil-loaded NPs on the Aβ fibril formation and their ability to cross the BBB was shown in a controlled release manner by using in vitro model (12).

Animals

Adult Sprague-Dawley (SD) male rats of 260–280 g weight were taken from Experimental Animals Unit at Hacettepe University. Animals were kept under 12-hour light/dark cycle with ad libitum access to food and water. All experimental procedures were approved by Hacettepe Local Animal Ethics Committee (permission number: 2012/13-10), and performed in compliance with national and local animal care and use guidelines.

Amyloid β Peptide

Amyloid β-Protein Fragment 25–35 (Sigma, Germany) was dissolved in distilled water (1 μg/1μL) and incubated in vitro at 37°C for 4 days. After the incubation, Aβ fibrils were injected ICV in accordance with the previous study (7). High hydrophobic 25–35 area of the amyloid β protein could form stable aggregates and cause neuronal death (13). Therefore, injection of $A\beta_{25-35}$ peptide to rat brain may cause time-dependent pathological changes (7).

Surgery and Drug Treatment

The surgical procedures were performed under anesthesia using a mix of 80 mg/kg ketamine and 10mg/kg xylazine. The animals were placed on a stereotaxic frame (Kopf Instruments, USA) with the tooth bar set to -2.3 mm. The coordinates for ICV injection were as follows: anteroposterior (AP)= -1 mm and mediolateral (ML)= ±1.5 mm calculated from bregma and dorsoventral (DV)= -3.5 mm from dural surface according to Paxinos and Watson rat brain atlas (14). 10μl $A\beta_{25-35}$ fibrils or saline (as a sham control) were injected at a rate of 0.4 μl/minute. The capillary glass was attached to the tip of Hamilton syringes to prevent the sticking of protein to the metal.

Experimental Groups

Experiments were divided into two parts:

1) Firstly, modeling Alzheimer's disease; $A\beta_{25-35}$ fibrils or saline were injected ICV to the rat brains (n=8/n=1). Animals were perfused transcardially with heparin followed by 4% paraformaldehyde (PFA) under a terminal dose of ketamine/xylazine anesthesia on the 23rd day. Brains were cut into 35 μm-thick coronal sections with a cryo-attachment sliding microtome (Leica SM2000 R Sliding microtome). Aβ staining was performed immunohistochemically to characterize the pathology.
2) Secondly, drug treatments group and behavioral examination;

Experimental groups;

1) 0.2 ml PBS were injected (IV) for 3 days (n=6, Aβ Alz group),
2) 0.2 ml donepezil (1 mg/kg) dissolved in PBS were injected (IV) for 3 days (n=6, free donepezil group),
3) 0.2 ml donepezil-loaded NPs (15 μg/kg) suspension in PBS were injected (IV) for 3 days (n=6, donepezil NPs group)
4) Naïve controls (n=6, control group)

After the stereotaxic Aβ injections; PBS, free donepezil, or donepezil NP treatments were applied three times for a week starting from 17th day and ending on 23rd day. While the IV injections continued, behavioral experiments started on 20th day and ended on 29th day. After all the behavioral tests were completed, brains were removed for the biochemical analyses (Figure 1).

Behavioral Tests

Animals were tested for motor and cognitive functions. All the tests were repeated at the same time of each day to prevent variation. Animals were kept in the experiment room for an hour to habituate and were handled by the same person for four consecutive days to decrease the level of anxiety before starting the behavioral tests. The schedule of the behavioral tests was arranged accordingly to minimize the stress caused by these tests (Figure 1).

Novel Object Recognition Test

Novel object recognition (NOR) is a cognitive function test for rats and mice. 40x40x40 cm square box and video tracking system (VideoMot2, Germany) were used. Animals' interactions and time spent with the objects were recorded by the system. For habituation, animals were put into empty boxes for 10 minutes in the first two days. On the third day, two identical objects (Brick blocks) were placed inside the boxes. Animals were allowed to get familiar with the objects for 10 minutes. For short-term memory,

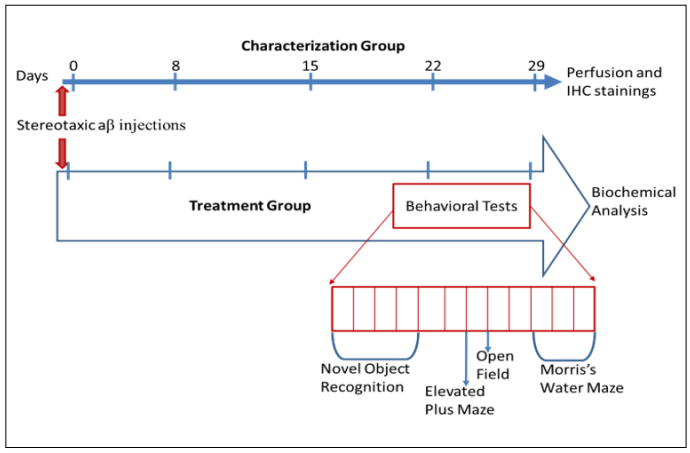

Figure 1. Schematic explanation of experimental procedure. Animals were divided into two groups; in the first group, Aβ Alz n=8; saline n=1; naïve control n=3, animals were perfused transcardially by 4% PFA for the immunostaining. In the second group; Aβ Alz n=6; donepezil n=6; donepezil PLGA-b-PEG NPs n=6; control n=6, animals were tested for behavioral changes and then without the perfusion brains were dissected for the biochemical analysis.

one hour after the familiarization phase, one of the objects was changed with a novel object, and animals were monitored for 5 minutes to detect the amount of time spent with the novel and the familiar objects. For long-term memory, 24 hours after the familiarization phase, the novel object is changed with an entirely different object (15). Animals were monitored once again for 5 minutes. In order to eliminate olfactory stimuli, boxes and objects were cleansed with 70% alcohol after each session. Results were stated as the ratio of (time spent with the novel object)/(time spent with the novel+familiar object) for each animal as a recognition index. Then, the mean ratio was calculated for each group for comparison (16).

Elevated Plus Maze (EPM)

Animals were tested for anxiety-like behavior on the EPM. Two of the opposite arms are open (open arm) and the other two opposite arms have 45 cm-high walls (closed arm). The room was kept dim and only illuminated by a desk lamp. The protocol described earlier was used (17). In short, animals were put into the center facing the closed arm and recorded for 5 minutes by the video tracking system (EthoVision XT, Netherlands). For each animal, value of time spent on the open arm/(time spent in the open arm + closed arm)x100 was calculated. Then, the average of these scores was calculated for each experiment group (17).

Open Field Locomotor Activity Test

Animals were tested for motor function with open field test. 50x50x30 cm boxes and the video tracking system (VideoMot2, Germany) were used. Total distance moved and horizontal activity was recorded for an hour and differences between the group means were analyzed (15).

Morris Water Maze (MWM)

Animals were tested for spatial learning and memory in MWM as described earlier (17). A circular, black-painted tank and a hidden platform were used. The tank was filled with 25°C tap water until the water covered the hidden platform. Some external clues were placed on the walls of the room for animals to be able to learn the location of the platform. Animals were placed into the tank, facing the wall of tank, for 4 consecutive days and recorded for 2 minutes with a video tracking system (VideoMot2, Germany). Each day, animals were put into the tank four times from different directions and the time they spent to find the platform was recorded. To create learning curves, each group's "time spent to find the hidden platform" was measured, and the differences between the groups were analyzed (16).

Immunohistochemistry

After the behavioral tests, animals were perfused transcardially with heparin followed by 4% PFA under high doses of ketamine/xylazine anesthesia. Brains were post-fixed with 4% PFA then transferred into 30% sucrose. Brains were cut into 35 µm-thick coronal sections with a cryo-attachment sliding microtome. Immunohistochemical staining was performed on free-floating sections. In short, initially, the sections were rinsed with Tris buffer solution (TBS) for three times, then pre-incubated with blocking solution for one hour. Sections were incubated overnight with the anti-amyloid beta primary antibodies (1:500, Abcam, UK) at room temperature. After rinsing the sections three times with TBS, they were incubated for 60 minutes in the biotinylated secondary antibody solution (Abcam, UK), then they were rinsed three times and incubated for 10 minutes with Biotin Streptavidin-peroxidase complex. Sections were developed by 3,3-diaminobenzidine as a chromogen (Abcam, UK), mounted on poly-l-lysine coated lamellas, dehydrated by gradually increasing the concentration of ethanol, rehydrated with xylene, and cover-slipped with DPX mounting medium. Slices were examined under light microscope (Leica DM4000B) attached with "NIS-Elements" imaging software (NIS-Elements Basic Research).

For the Congo red staining; sections were mounted on poly-l-lysine coated lamellas and dried, then incubated with Congo red staining for 90 minutes. They were rinsed few times with distilled water. After being incubated with modified Mayer solution for 3 minutes, they were washed and rehydrated with xylene for 5 minutes, then dehydrated by gradually increasing ethyl alcohol solutions for 1-2 minutes and cover-slipped with DPX. Slices were examined through light microscope (Leica DM4000B) attached with "NIS-Elements" imaging software (NIS-Elements Basic Research).

As with the Congo red staining method, Thioflavin S was applied to the mounted sections. 1% thioflavin S solution was prepared in distilled water and then filtered. Sections were dehydrated with xylene, and then hydrated in gradually decreasing ethyl alcohol solutions for 1–2 minutes. Sections were dipped into the water a few times and stained with Thioflavin S for 30 minutes. After being dehydrated with gradually increasing ethyl alcohol solutions for 1–2 minutes, they were rehydrated with xylene for 5 minutes once more, and cover-slipped with DNA staining compound Hoechst (Thermo Scientific). Slices were examined under fluorescence microscope attached with "NIS-Elements" imaging software (NIS-Elements Basic Research).

Determination of Acetylcholinesterase Activity in Brain Homogenates

Total protein concentration in the supernatants of brain homogenates was measured following the instructions of the BCA protein assay kit (Intron Biotechnology).

Acetylcholinesterase activity measurements were performed at 25°C in 500 mM potassium phosphate buffer (pH 7.4) containing 0.125 mM dithiobisnitrobenzoic acid and 5 mM acetylthiocholine (ATC) as substrate (18). Reactions were initiated by adding enzyme and monitored using spectrophotometer at 412 nm. ε=14.β mM^{-1} cm^{-1} value and total protein amount of brain homogenates were used in the calculations. Acetylcholinesterase activity is shown in nmol/min/mg.

Statistical Analyses

Experimental data were presented as the mean ± standard error of the mean (SEM). By using GraphPad prism 7, one-way analysis of variance (ANOVA), then Dunnett post hoc analysis was used for analysis, and Kruskal-Wallis followed by Dunn's multiple comparison tests were applied only for MWM analysis. Statistical significance was set at $P<0.05$.

RESULTS

Amyloid β Injection Into the Lateral Ventricle Leads To Morphological Changes In Hippocampus

Aβ immunohistochemical staining was performed to confirm the pathology occurring as a result of $A\beta_{25-35}$ peptides injection (A_β Alz, n=6). Aβ fibril, congo red, and thioflavin S staining was observed especially around the vessels and the ventricle, dentate gyrus of hippocampus, entorhinal cortex, perivascular and parenchymal tissue of cortex (Figure 2a-c). In addition, we observed neurofibrillary tangle-like accumulations in cortex parenchyma after thioflavin S staining (Figure 2c). However, no staining was observed in saline injected group (Figure 2a).

Amyloid β Injection Into the Lateral Ventricle Leads To Behavioral Changes

Animals were assessed by NOR and MWM tests for the cognitive function. In our test; Aβ Alz group spent less time to explore the novel object in comparison to naïve control group one hour after the training session (F=9.28; P=0.0069 Aβ Alz vs Naïve; Figure 3a), indicating noticeable short-term memory deficit. Donepezil-loaded PLGA-b-PEG NPs (Donepezil NPs) group spent more time with the novel object but the free donepezil (Donepezil) group spent less time with the novel object one hour after training session (F=9.28; P=0.0018 Aβ vs Donepezil NPs; Figure 3a). These results showed short-term memory impairment after $A\beta_{25-35}$ injection, which improved with donepezil NPs treatment. In order to analyze the long-term memory, we repeated the same protocol by changing one of the familiar objects with a different object 24 hours after the training session, however, there was no difference between the groups (Figure 3b).

MWM test was applied to analyze spatial learning and memory. The animals were trained to find the hidden platform for four consecutive days and the time they reach the platform was recorded (Figure 3c). All the groups showed a progress in finding the hidden platform with each passing day, except the Aβ Alz group (4th day average time of Aβ Alz=16.92; Donepezil NPs=10.25; Donepezil=11.45; Naïve controls=12.625, Figure 3c). On the 4th day, Aβ Alz group spent more time to find the platform, but the increase was statistically significant only in comparison to donepezil NPs group (F=4.264; P=0.0238, Aβ Alz vs Donepezil NPs, Figure 3d). Yet, both donepezil NPs and free donepezil groups spent less time to find the platform compared to Aβ Alz group. The time spent in targeted quadrant vs. all quadrants, with a slight increase in donepezil NPs, was almost equal between all groups (Figure 3e). The velocity was also similar between all

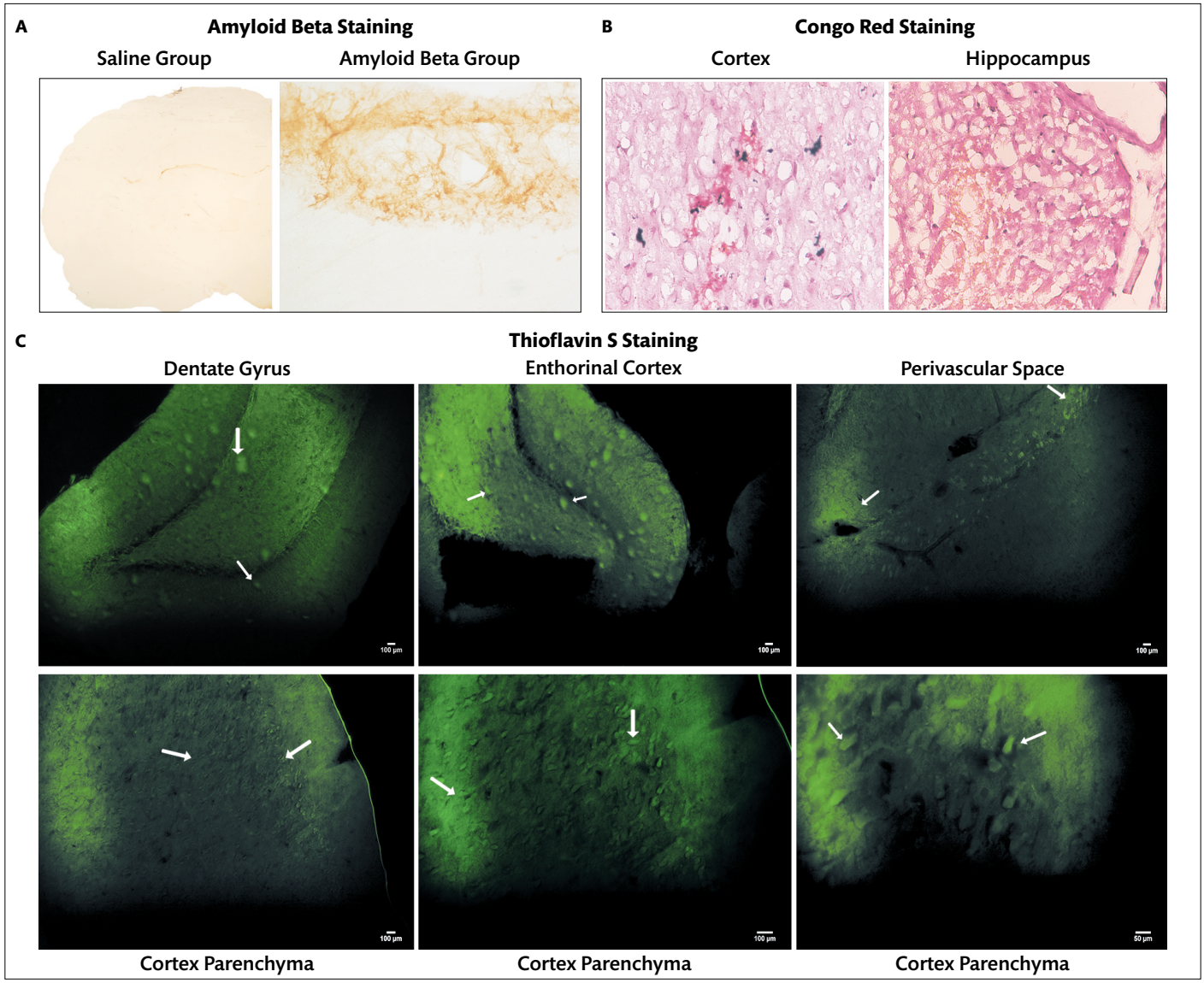

Figure 2. a–c. Histopathological changes due to Amyloid β injection. Representative sections of amyloid β fibril staining of Saline and Amyloid β injected Alz group **(a).** Representative sections from hippocampus and cortex stained with Congo red **(b).** Representative sections of thioflavin S staining in dentate gyrus, entorhinal cortex, and perivascular space at 10x (in first row); cortex parenchyma at 10x, 20x, and 40x (in second row) magnifications, respectively (scalebars=100 in first row, 100, 100 and 50 µm in second row, respectively) **(c).**

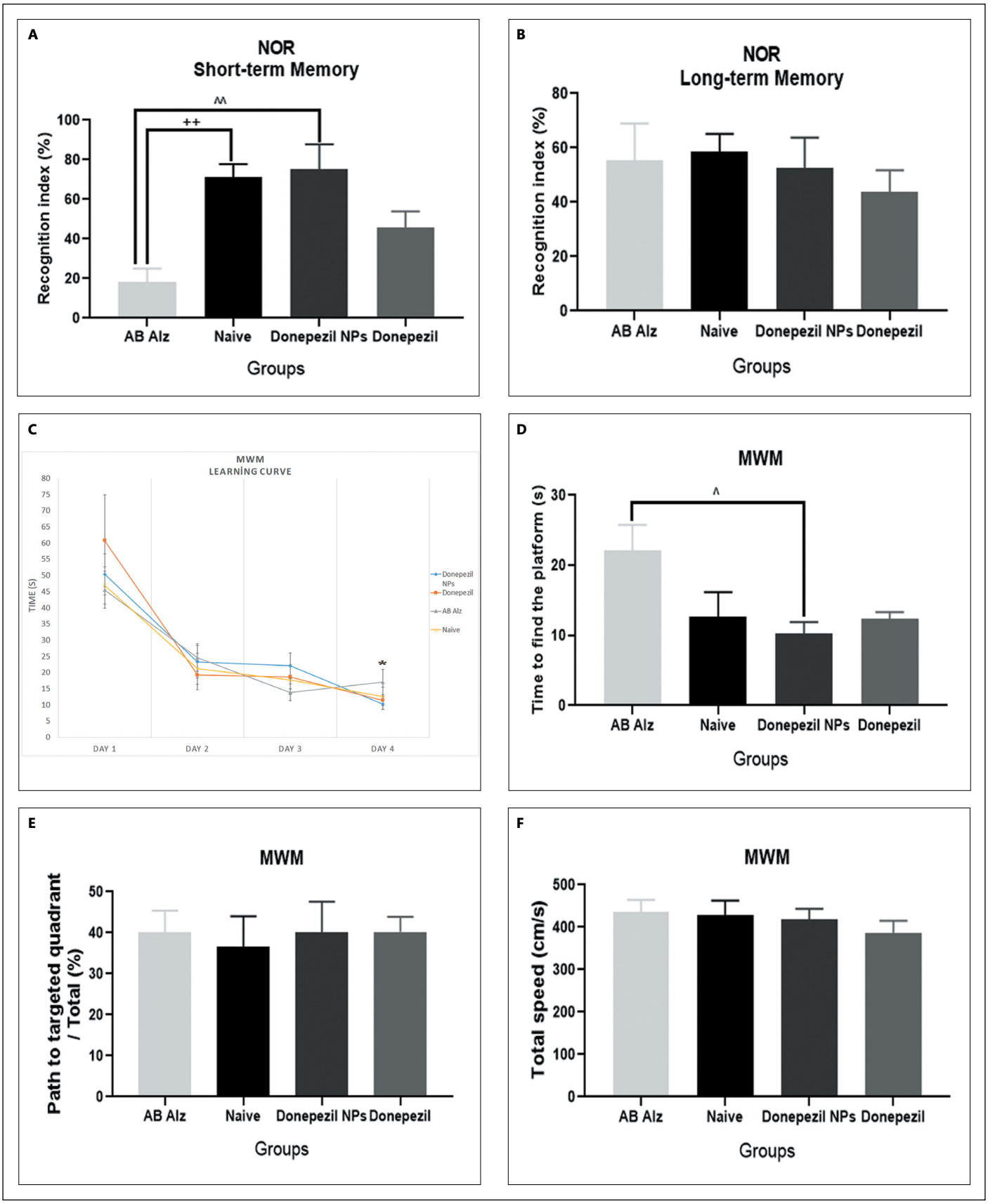

Figure 3. a–f. Amyloid β injection into the lateral ventricle leads to learning and memory deficits and donepezil PLGA-b-PEG NPs treatment is able to reverse it. Recognition index (%) in NOR test after 1-hour was significantly lower in the Aβ Alz group compared to naïve controls indicating a short-term memory deficit and donepezil NPs treatment reversed the memory deficits **(a)** (+P<0.05 Aβ Alz vs naïve, ^p<0.05 Aβ Alz vs Donepezil NPs; one-way ANOVA followed by Dunnett post hoc analysis). There was no change between the groups after 24 hours in NOR test **(b).** Learning curves of groups in MWM test; naïve controls and treatment groups find the hidden platform before Aβ Alz group during the 4-day learning phase **(c).** The last day of the trials, time spent to find hidden platform was significantly longer with Aβ Alz group **(d)** (^P<0.05 Aβ Alz vs Donepezil NPs, Kruskal-Wallis followed by post hoc Dunn's multiple comparison test). Distance moved in the targeted platform to all quadrants **(e)** and the velocity was similar between all groups **(f)** (n=6 Aβ Alz, n=6 Donepezil, n=6 Donepezil PLGA-b-PEG NPs, n=6 naïve control, Values represent mean ± SEM; NOR: novel object recognition, MWM: Morris's Water Maze)

groups (Figure 3f), indicating that the observed difference is not due to motor impairment. These results pointed out spatial memory impairment after $A\beta_{25-35}$ injection, which improved with donepezil NPs.

Animals were tested for anxiety-like behaviors using EPM. Aβ Alz group spent less time on the open arm relative to naïve controls (F=9.65; P=0.0312 Aβ Alz vs Naïve; Figure 4A). Free donepezil group spent less time on the open arm just as Aβ Alz without statistical significance (Figure 4a). Donepezil NPs group spent more time on the open arm yet this tendency did not reach statistical significance (Figure 4A). Both Aβ Alz and free donepezil groups, but not the donepezil NPs treatment group, enter the open arm less than naïve controls (F=7.95; P=0.0033 Aβ Alz vs Naïve, P=0.0239 Donepezil vs Naïve, Figure 4b). Aβ Alz and free donepezil groups spent slightly more time on closed arms without a statistical significance (Figure 4c). These results remarked that $A\beta_{25-35}$ injection led to anxiety and donepezil NPs treatments were more effective against anxiety compared to free donepezil treatment.

Locomotor activity test was applied to examine the motor function. Aβ Alz group distance moved was similar to naïve control group (Figure 4d). However, both free donepezil and donepezil NPs treatments reduced movement. Yet, only the decrease in free donepezil group was statistically significant compared to Aβ Alz and naïve control groups (F=5.798; P=0.04 Aβ Alz vs Donepezil, P=0.0085 Donepezil vs Naive; Figure 4d). Donepezil NPs treatment slightly improves the locomotion compared to free donepezil treatment without presenting a statistical significance (Figure 4d). These results showed that $A\beta_{25-35}$ injection did not affect the motor movement but free donepezil decreased the distance moved.

Donepezil-loaded Nanoparticles Treatment Has a Stronger Effect in Inhibition of Acetylcholinesterase Activity

Acetylcholinesterase activity levels are given in Table 1 (nmol/min/mg). When study groups were evaluated, an increase in acetylcholinesterase activity was observed due to the presence of Aβ (Aβ Alz vs Naïve, P<0.01,

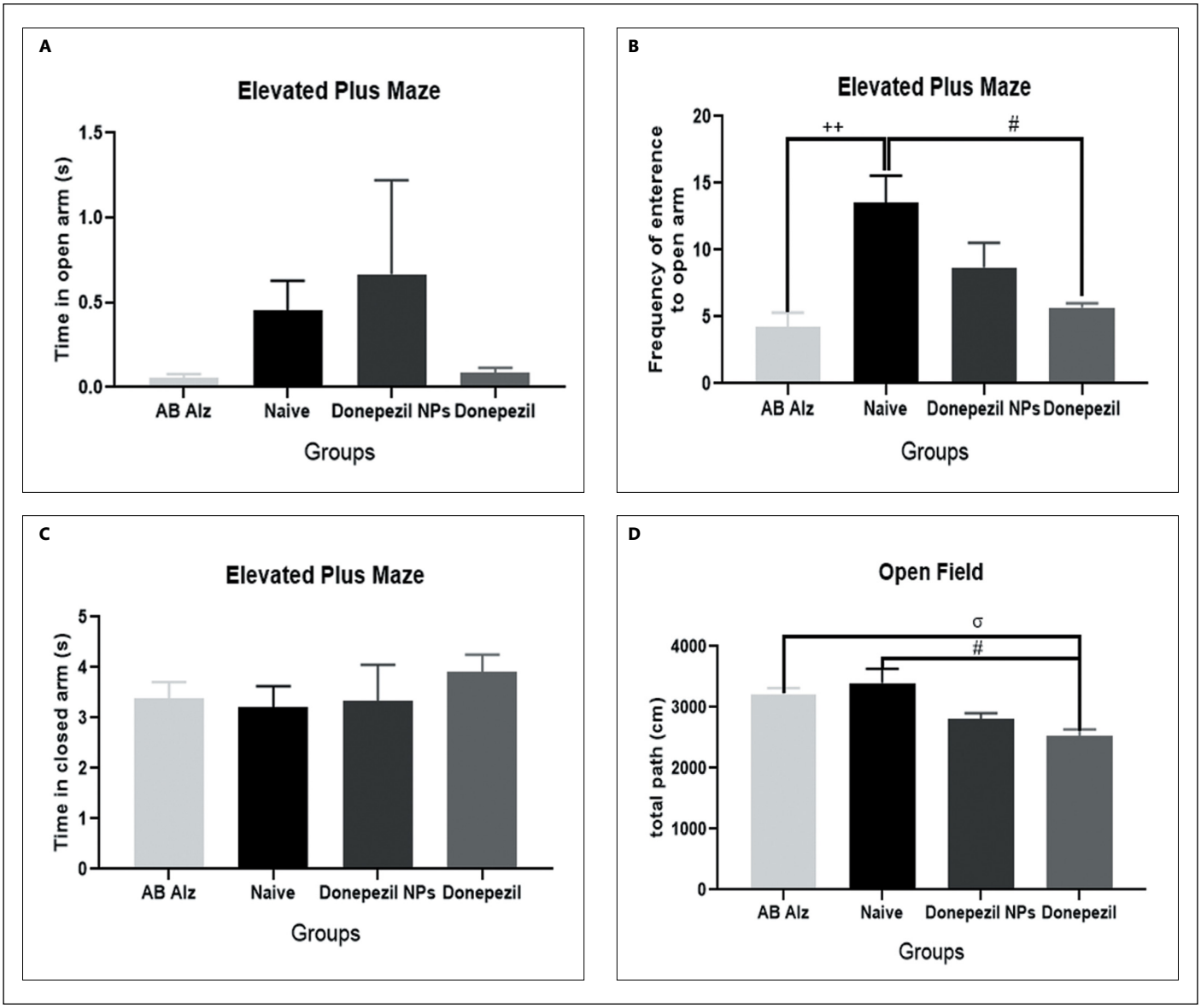

Figure 4. a–d. Amyloid β injection into the lateral ventricle leads to anxiety-like behavior and donepezil PLGA-b-PEG NPs treatment improved it. Time spent in the open arm was the lowest in Aβ Alz group and was highest in donepezil NPs group in EPM test **(a).** Aβ Alz group and free donepezil group less frequently enter the open arm than naïve controls **(b)** (+P<0.05 Aβ Alz vs naïve, # P<0.05 Donepezil vs naïve; one-way ANOVA followed by Dunnett post hoc analysis), but for the time spent in the closed arm; there were no differences between the groups **(c).** In the open field locomotor activity test; total path was distinctively lower in the free donepezil group compared to Aβ Alz and naïve controls group **(d)** (σP<0.05 Donepezil vs Aβ Alz, #P<0.05 Donepezil vs naïve control; one-way ANOVA followed by Dunnett post hoc analysis; n=6 Aβ Alz, n=6 Donepezil, n=6 Donepezil PLGA-b-PEG NPs, n=6 naïve control, Values represent mean ± SEM; EPM: elevated plus maze).

Table 1. Acetylcholinesterase activity levels in control and experimental groups

Study Groups	Acetylcholinesterase activity (nmol/dk/mg)
Aβ Alz**	74.460 ± 13.653
Naïve	50.452 ± 5.538
Donepezil NPsΨΨ	53.114 ± 8.778
Donepezil (Free)	57.798 ± 11.224

**P<0.01 Aβ Alz vs. Naïve.
ΨΨ P<0.01 Donepezil NPs vs. Aβ Alz.
Aβ Alz: Alzheimer group; NPs: Nanoparticles.

Table 1). This increase in enzyme activity returned to normal levels with the application of free donepezil and donepezil NPs treatments. Additionally, donepezil NPs were found to be more effective than free donepezil (P<0.01, Table 1). This was due to the controlled release profile of the drug with NPs.

DISCUSSION

AD is a progressive neurodegenerative disease, the most common type of dementia, and caused by irreversible neuronal loss in the hippocampus and cortex (1). There is still no definitive treatment for AD and drugs like donepezil can be used only for symptomatic treatment despite their side effects and compliance problems.

Previously, donepezil-loaded PLGA-b-PEG NPs were synthesized, characterized, and optimized for IV or intraperitoneal administration, and targeted to amyloid fibrils in order to minimize tissue distribution and maximize local concentrations in the targeted area (12). The results showed that donepezil-loaded NPs destabilize the Aβ fibril formation in vitro and have the ability to cross the BBB and also have a neuroprotective effect against Aβ fibrils (12). As a matter of fact, these findings are consistent with the literature that PEGylated NPs have longer duration of action, better bioavailability, and improve the treatment by targeting the Aβ in vivo (19).

In this study, pre-aggregated $A\beta_{25-35}$ was injected intracerebroventricularly to establish the AD pathology and this resulted in mild cognitive decline. There was no neuronal loss in the cortex or hippocampus, yet, behavioral changes were observed. Therefore, it was suggested that Aβ fibrils lead to memory and learning impairment due to synaptic dysfunction without the neuronal loss (20). So, this $A\beta_{25-35}$ fibril rat model is beneficial to model mild Alzheimer's disease and study the cognitive functions due to the observed behavioral changes.

Short-term and long-term memory was tested with NOR test. The short-term memory impairment was observed in Aβ Alz group, which was reversed by donepezil-loaded NPs treatment. Although brain-targeted donepezil by NPs were administered in much lower dose than IV free donepezil, it improved short-term memory more significantly than free donepezil group, which shows that donepezil's direct targeting of the brain establishes a significant opportunity for treatment (21,22). However, for the long-term memory, no differences were observed between the Aβ Alz and treatment groups. All groups spent almost equal time with both objects, indicating that after 24 hours, animals had difficulties in remembering, therefore, they were interested in both objects equally (23).

Spatial learning and memory were tested by MWM test. It showed that Aβ Alz group was not able to learn the location of the hidden platform and donepezil-loaded NPs treatment improve the spatial memory. While both free donepezil and donepezil-loaded NPs groups found the hidden platform similar manner to naïve controls, only donepezil-loaded NPs treatment significantly shortened the time to find the platform compared to Aβ Alz group. This demonstrates that targeted NPs are more successful (21,22).

The EPM test was performed to examine anxiety-like behavior in rats. While anxiety-like behavior was observed in the Aβ Alz group, administration of free donepezil did not change this behavior. In contrast, donepezil-loaded NPs treatment reduced the anxiety-like behavior. Induction of anxiety is a well-known effect of the donepezil (24,25). However, donepezil-loaded NP group had a standard error higher than other groups, effects similar to hypo-anxiety was observed. When the frequency of entrance to open arm was examined, it was observed that both donepezil treatment groups entered less than naïve controls and spent more time on the closed arm. So, the reason for donepezil-loaded NPs spending longer time on the open arm could be due to freezing reflex. In addition, if the total length of paths are taken into consideration, both treatment groups moved less in total which also indicates freezing reflex and anxiety-like behavior (24,26).

The open field test was performed to investigate the locomotor activity and to ensure that the changes are not due to motor impairment. In our experiments, Aβ Alz group moved as much as naïve controls. However, both of the donepezil-treated groups moved less, especially the free donepezil group. It is known that donepezil can reduce the spontaneous locomotion and lead to anxiety-like behavior (24,25), and if the animals have anxiety, they tend to stay on the dark edges rather than the center and the free donepezil group spent most of their time in the closed arm and avoided entering the open arm. In addition, MWM test showed no significant difference in total speed or total distance moved between the groups. All of these suggest that anxiety could be the reason, rather than motor dysfunction.

In order to compare the efficacy of free donepezil and donepezil-loaded NPs following in vivo experiments, acetylcholinesterase activity was determined. Specific activity in the naïve group was determined as 50.452 nmol/min/mg protein, which is consistent with the literature (27). It was determined that the activity increased significantly in the Aβ Alz group. While IV administration of free donepezil reduces the activity; IV application of donepezil-loaded NPs decreased the activity more than free donepezil did. Based on these results, it can be said that the ability of donepezil-loaded NPs to inhibit acetylcholinesterase activity is higher than free donepezil. This can be considered as a result of the controlled release of donepezil from NPs and its longer effective period. Results showed that Aβ Alz group acetylcholinesterase activity was increased compared to naïve controls. While acetylcholinesterase activity is expected to decrease in AD due to cholinergic terminal loss, several studies are showing that acetylcholinesterase activity can be increased in relation to Aβ (28,29). The different forms of acetylcholinesterase could be one of the reasons for this acetylcholinesterase activity increase, according to a study which showed an increase in monomeric form of

the acetylcholinesterase in the brain after ICV injection of the Aβ (30). In another study, it has been shown that acetylcholinesterase presence in the critical locations also have a role in triggering Aβ toxicity by inducing new oligomer and fibril formation (28, 29). Even though the mechanism is not clear, these data could explain the increase in acetylcholinesterase activity seen in Aβ Alz group and how the donepezil treatment decrease this activity. All these results indicate that donepezil-loaded NPs treatment is more effective and promising due to direct targeting of brain and longer period of effectiveness.

In conclusion, we showed that donepezil-loaded NPs treatment increases the learning and memory function, and inhibits the acetylcholinesterase activity in this Aβ fibril model of AD. Therefore, drug-targeting treatment via NPs could be a promising therapy opportunity. For the further studies, testing long-term chronic effects of donepezil-loaded NPs in vivo may offer a better perspective.

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REFERENCES

1. Adlard PA, Tran BA, Finkelstein DI, Desmond PM, Johnston LA, Bush AI, et al. A review of beta-amyloid neuroimaging in Alzheimer's disease. Front Neurosci. 2014;8:327. PubMed PMID: 25400539. Pubmed Central PMCID: PMC4215612. Epub 2014/11/18. [Crossref]
2. Ballard C, Gauthier S, Corbett A, Brayne C, Aarsland D, Jones E. Alzheimer's disease. Lancet. 2011;377(9770):1019–1031. [Crossref]
3. Barage SH, Sonawane KD. Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease. Neuropeptides. 2015;52:1–18. PubMed PMID: 26149638. Epub 2015/07/08. [Crossref]
4. Checler F, Vincent B. Alzheimer's and prion diseases: distinct pathologies, common proteolytic denominators. Trends Neurosci. 2002;25(12):616–620. PubMed PMID: 12446128. Epub 2002/11/26. [Crossref]
5. Hampel H, Mesulam M-M, Cuello AC, Farlow MR, Giacobini E, Grossberg GT, et al. The cholinergic system in the pathophysiology and treatment of Alzheimer's disease. Brain. 2018;141(7):1917–1933. PubMed PMID: 29850777. Pubmed Central PMCID: PMC6022632. Epub 2018/06/01. [Crossref]
6. Yamada K, Nabeshima T. Animal models of Alzheimer's disease and evaluation of anti-dementia drugs. Pharmacol Ther. 2000;88(2):93–113. PubMed PMID: 11150591. Epub 2001/01/11. [Crossref]
7. Zussy C, Brureau A, Delair B, Marchal S, Keller E, Ixart G, et al. Time-course and regional analyses of the physiopathological changes induced after cerebral injection of an amyloid beta fragment in rats. Am J Pathol. 2011;179(1):315–334. PubMed PMID: 21703413. Pubmed Central PMCID: PMC3123848. Epub 2011/06/28. [Crossref]
8. Pottiez G, Duban-Deweer S, Deracinois B, Gosselet F, Camoin L, Hachani J, et al. A differential proteomic approach identifies structural and functional components that contribute to the differentiation of brain capillary endothelial cells. J Proteomics. 2011;75(2):628–641. PubMed PMID: 21982828. Epub 2011/10/11. [Crossref]
9. Simşek S, Eroğlu H, Kurum B, Ulubayram K. Brain targeting of Atorvastatin loaded amphiphilic PLGA-b-PEG nanoparticles. J Microencapsul. 2013;30(1):10–20. PubMed PMID: 22734433. Epub 2012/06/28. [Crossref]
10. Yetisgin AA, Cetinel S, Zuvin M, Kosar A, Kutlu O. Therapeutic Nanoparticles and Their Targeted Delivery Applications. Molecules. 2020;25(9):2193. PubMed PMID: 32397080. Pubmed Central PMCID: PMC7248934. Epub 2020/05/14. [Crossref]
11. Takada-Takatori Y, Nakagawa S, Kimata R, Nao Y, Mizukawa Y, Urushidani T, et al. Donepezil modulates amyloid precursor protein endocytosis and reduction by up-regulation of SNX33 expression in primary cortical neurons. Sci Rep. 2019;9(1):11922. PubMed PMID: 31417133. Pubmed Central PMCID: PMC6695423. Epub 2019/08/17. [Crossref]
12. Baysal I, Ucar G, Gultekinoglu M, Ulubayram K, Yabanoglu-Ciftci S. Donepezil loaded PLGA-b-PEG nanoparticles: their ability to induce destabilization of amyloid fibrils and to cross blood brain barrier in vitro. J Neural Transm (Vienna). 2017;124(1):33–45. PubMed PMID: 26911385. Epub 2016/02/26. [Crossref]
13. Pike CJ, Walencewicz-Wasserman AJ, Kosmoski J, Cribbs DH, Glabe CG, Cotman CW. Structure-activity analyses of beta-amyloid peptides: contributions of the beta 25–35 region to aggregation and neurotoxicity. J Neurochem. 1995;64(1):253–265. PubMed PMID: 7798921. Epub 1995/01/01. [Crossref]
14. Paxinos G, Watson C. The rat brain in stereotaxic coordinates –hard cover edition. 6th Ed. US: Elsevier, Academic Press; 2006.
15. Uğur Mutluay S, Çınar E, Yalçın Çakmaklı G, Ulusoy A, Elibol B, Tel BC. Modelling Non-motor Symptoms of Parkinson's Disease: AAV Mediated Overexpression of Alpha-synuclein in Rat Hippocampus and Basal Ganglia [Parkinson Hastalığında Motor Dışı Semptomların Modellenmesi: Sıçan Hipokampüsünde ve Bazal Gangliyonlarında Alfa-sinukleinin AAV Aracılı Aşırı İfadesi]. Turk J Neurol. 2020;26(4):322–329. [Crossref]
16. Tel BC, Kazkayası İ, Telli G, Cinar E, Mutluay SU, Çakmaklı GY, et al. Higher alpha-synuclein aggregate density does not lead to more severe dopaminergic cell loss in the AAV-mediated overexpression model of Parkinson's Disease: A timecourse study. J Res Pharm (online). 2021;25(3):352–358. [Crossref]
17. Cinar E, Yalcin-Cakmakli G, Saka E, Ulusoy A, Yuruker S, Elibol B, et al. Modelling cognitive deficits in Parkinson's disease: Is CA2 a gateway for hippocampal synucleinopathy? Exp Neurol. 2020;330:113357. PubMed PMID:32437708. Epub 2020/05/22. [Crossref]
18. Ellman GL, Courtney KD, Andres V, Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7:88–95. PubMed PMID:13726518. Epub 1961/07/01. [Crossref]
19. Shadab MD, Ali M, Baboota S, Sahni JK, Bhatnagar A, Ali J. Preparation, characterization, in vivo biodistribution and pharmacokinetic studies of donepezil-loaded PLGA nanoparticles for brain targeting. Drug Dev Ind Pharm. 2014;40(2):278–287. PubMed PMID:23369094. Epub 2013/02/02. [Crossref]
20. Tu S, Okamoto S, Lipton SA, Xu H. Oligomeric Abeta-induced synaptic dysfunction in Alzheimer's disease. Mol Neurodegener. 2014;9:48. PubMed PMID:25394486. Pubmed Central PMCID. PMC4237769. Epub 2014/11/15. [Crossref]
21. Teleanu DM, Chircov C, Grumezescu AM, Volceanov A, Teleanu RI. Impact of Nanoparticles on Brain Health: An Up to Date Overview. J Clin Med. 2018;7(12):490. PubMed PMID:30486404. Pubmed Central PMCID. PMC6306759. Epub 2018/11/30. [Crossref]
22. Yoo JH, Valdovinos MG, Williams DC. Relevance of donepezil in enhancing learning and memory in special populations: a review of the literature. J Autism Dev Disord. 2007;37(10):1883–1901. PubMed PMID:17221321. Epub 2007/01/16. [Crossref]
23. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cognitive processing. 2012;13(2):93–110. PMID: 22160349 PMCID: PMC3332351. [Crossref]
24. Giacomini AC, Bueno BW, Marcon L, Scolari N, Genario R, Demin KA, et al. An acetylcholinesterase inhibitor, donepezil, increases anxiety and cortisol levels in adult zebrafish. J Psychopharmacol. 2020;34(12):1449–1456. PubMed PMID: 32854587. Epub 2020/08/29. [Crossref]
25. Pompeia S, Gouveia JR, Galduroz JC. Acute mood effect of donepezil in young, healthy volunteers. Hum Psychopharmacol. 2013;28(3):263–269. PubMed PMID: 23653426. Epub 2013/05/09. [Crossref]
26. Pavlova IV, Rysakova MP, Zaichenko MI, Broshevitskaya ND. Behavior of rats with high and low levels of freezing in defensive situations and on selection of food reinforcement. Neurosci Behav Physiol. 2020;50(1):126–136. [Crossref]

27. Tang J, Carr RL, Chambers JE. Changes in rat brain cholinesterase activity and muscarinic receptor density during and after repeated oral exposure to chlorpyrifos in early postnatal development. Toxicol Sci. 1999;51(2):265–272. PubMed PMID: 10543028. Epub 1999/10/30. [Crossref]
28. Mesulam M. The cholinergic lesion of Alzheimer's disease: pivotal factor or side show? Learn Mem. 2004;11(1):43–49. PubMed PMID: 14747516. Epub 2004/01/30. [Crossref]
29. Carvajal FJ, Inestrosa NC. Interactions of AChE with Abeta Aggregates in Alzheimer's Brain: Therapeutic Relevance of IDN 5706. Front Mol Neurosci. 2011;4:19. PubMed PMID: 21949501. Pubmed Central PMCID: PMC3172730. Epub 2011/09/29. [Crossref]
30. Saez-Valero J, de Ceballos ML, Small DH, de Felipe C. Changes in molecular isoform distribution of acetylcholinesterase in rat cortex and cerebrospinal fluid after intracerebroventricular administration of amyloid beta-peptide. Neurosci Lett. 2002;325(3):199–202. PubMed PMID: 12044655. Epub 2002/06/05. [Crossref]