Original Article

Possible Preventive Effect of Donepezil and Hyoscyamoside by Reduction of Plaque Formation and Neuroinflammation in Alzheimer's Disease

Abstract

Background: Alzheimer disease (AD) is the most common age-dependent dementia. The complex natural accumulation of amyloid beta (Aβ) precursor protein in hippocampus neurons is regarded as the earliest pathological feature of AD, although there are cholinergic assumptions and effective inflammation in AD. In this animal experimental study, we evaluated the preventive effect of hyoscyamoside (Hyo) and donepezil (Dz) on plaque formation and improvement of neurogenic inflammation in AD rats. **Methods:** Dz was prepared and Hyo (steroidal saponin) was isolated from *Hyoscymus niger*. Then, Wistar rats divided into five groups including negative and positive controls, AD, Dz, and Hyo treatment groups based on the drug exposure and their behavioral alternation was examined using Morris water maze (MWM) test. Bielschowsky staining was used to detect the nerve fibers. Serum levels of interleukin (*IL*)-4 and *IL-6* were evaluated by ELISA. The RNA expression of cyclin-dependent kinase *CDK11-P58* in peripheral blood lymphocytes was performed using quantitative PCR. **Results:** The MWM test showed significant changes in time the models spent to find the hidden platform. The Hyo treatment group showed a notable speed change ($P < 0.01$). The histopathological analysis of the hippocampal tissue revealed the inhibition of Aβ formation in the treatment groups. The treatment groups had a significant decline in the serum level of *IL-6*, and the *IL-4* serum level was increased in the Hyo and Dz treated groups. The expression levels of *CDK11-P58* was significantly decreased in the treatment groups. **Conclusions:** In sum, the therapeutic effects of Hyo is comparable with that of Dz in AD rats by suppressing neuroinflammation. Thus, these compounds could be considered as a preventive agent in the AD therapy.

Keywords: *Alzheimer disease, amyloid beta-protein, donepezil, hyoscyamoside, neurogenic inflammation*

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Introduction

Alzheimer disease (AD) is the most common type of dementia among aged people presenting in mid- to late adult life. This neurodegenerative disorder is known to cause progressive defects in memory, cognition, and lack of motor planning to perform tasks and movements and impaired reasoning.[1] The manifestation of AD includes "early-onset" and "late-onset" forms and familial AD occurs mostly in the early-onset type.[1] The prevalence of the disease varies from 0.6% in 65-year-old people to 10% in people aged 80 or above.[2]

Alzheimer's assumptions include beta-amyloid (Aβ) plaque, inflammation, and cholinergic. Aβ accumulation results in neurofibrillary tangles of hyperphosphorylated microtubule associated tau protein via the activation of *(GSK3β)* by enhancing Ras/ERK signaling cascade and neuroinflammation development.[3] While normal Tau protein stabilizes the microtubules in neurons, the hyperphosphorylated form of the protein has reduced binding ability to microtubules. Aggregation of Aβ leads to glutamate neurotoxicity either by excess secretion of glutamate neurotransmitters or inhibition of glutamate receptors.[4] Aβ senile plaques attract and activate microglia which are the phagocytes present in the central nervous system. They respond to the aggregation of pathogens in the brain and trigger neuroinflammation and oxidative stress by secretion of cytokines and reduction of cognition.[5] According to this theory, the reduction in acetylcholine content due to the release of excess acetylcholinesterase (AchE) plays an important role in Alzheimer's memory. The stimulation of cholinergic receptors would inhibit the accumulation of Aβ plaques and hyperphosphorylated tau

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protein[6] resulting in the aggregation of insoluble Aβ peptides which has a destructive effect on neurons and mitochondria.[5] Since the low expression of β-site amyloid precursor protein) APP (cleaving enzyme (BACE1) leads to a decrease in the production of Aβ, this molecule could be involved in amyloid plaque formation.[4]

The cyclin-dependent kinase (*CDK*)-*11* genes comprise of numerous splice variant transcripts, the main isoforms of which are *P110* and *P58*.[7] Their play important role in re-entering the cell cycle in a neuron of AD, thus providing a fascinating new function of the APP signal pathway in AD.[7]

The response of proinflammatory cytokines, notably *IL-1α*, *IL-1β*, tumor necrosis factor alpha *(TNF-α)*, and interferon gamma *(INF-γ)* have been found in the brain autopsy analysis of AD patients.[8] Senile plaques can cause loss of cholinergic activity in the central nervous system of AD patients due to their toxic effect on the cholinergic receptors. Experimental studies revealed the role of acetylcholine in memory and cognition. Blocking the cholinergic activity of young subjects leads to memory impairments like those seen in aged individuals. It is noteworthy that phenotypes can be reversed by a cholinergic agonist.[6]

Dz, an AChE inhibitor (AChEI) acts by blocking AChE and increasing the persistence of synaptic acetylcholine.[9]

Hyoscyamoside is a natural product of *Hyoscyamus niger*, Solanaceae family. It could be used for the treatment of inflammation diseases, respiratory diseases such as bronchitis and rheumatism, and motion sickness such as multiple sclerosis.[10]

Hyoscyamoside is a steroidal saponin(glycoside) containing glycosides (A–G) and is derived from tigogenin, diosgenin and forest, respectively that ameliorates cognitive deficits of the brain, induces memory and reduces oxidative damages.[11] Saponins are a group of glycosides with surface active compounds including steroids with anti-inflammatory activities. Some herbs have been established to possess phosphodiesterase activity owing to their saponin content.[12] Experiments revealed that diosgenin acts as an anti-inflammatory agent through change of *IL-4* and *IL-6* expression.[13,14] Medicinal herbs containing saponins have anti-inflammatory actions through inhibition of *TNF-α*, *IL-1β*, and *COX-2*.[12]

In the present study, we tried to evaluate the possible preventive effect of Dz and hyoscyamoside (Hyo) in relieving plaque formation and reducing neurogenic inflammation in AD rats.

Methods

Sample and extract preparation

Alzheimer's disorder was induced via injection of 2 μL Aβ1-42 (Sigma Aldrich) into both the CA1 regions of the hippocampus using a Paxinos atlas.[15]

Dz was administered (4 mg/kg/day) through oral gavage with normal saline obtained from the Loghman Pharmaceutical Co., Tehran, I.R. Iran. The *Hyoscymus niger*, commonly known as Henbane, is identified as the source of steroidal saponins. Hyo (steroidal saponins) (10 mg/kg/day) was extracted via phytochemical methods[16] and administered through oral gavage with normal saline (0.9%).

Firstly, extracted using methanol by Soxhlet method. Then, chloroform and isopropanol were added, then hyoscyamoside were separated by chloroform.[17,18]

Animal experiments

In our study, adult male Wistar rats ($n = 50$, 180 g) were purchased from the Pasteur Institute of Iran, Tehran, I.R. Iran. Rats were stored in five groups (22°C, humidity of 30–40%, and 12/12-h light/dark). They had free access to food and water in the cages. All the experiments were performed between 9 AM to 4 PM and randomly divided into five groups of 10. The first group, negative control, received normal diet with no surgical experiment. The second group, positive control, contains animals with surgical experiment and received PBS. Positive and negative controls were approximately equal and negative control was considered as the control group. The third group, the AD group, contains animals receiving Aβ after 20 days of inducing Alzheimer's and receiving 1 CC of (n/saline) per day for 28 days. The fourth group contains animals receiving Aβ after 20 days of inducing Alzheimer's and also receiving 4 mg/kg/day of Dz orally using oral gavage for 28 days. The fifth group contains animals receiving Aβ after 20 days of inducing Alzheimer's and receiving 10 mg/kg/day of Hyo administered orally using oral gavage for 28 days.

Morris water maze test

Spatial learning and cognitive function were examined via Morris water maze (MWM) test after completion of treatment at day 28. The maze was a round black pool (with 180 cm diameter and 60 cm height) filled with warm water (22°C) to a depth of 30 cm. A hidden escape platform of 10 cm diameter was embedded 5 cm under the surface of the water at a fixed position. The pool was divided into four identical spaces and the escape platform was located at the center of one of the quadrants. The amount of time spent finding the platform, and the swimming speed were measured. The experiments were conducted four times per day in four consecutive days.

Histopathological analysis

The rats were first anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (10 mg/kg). The cerebral hippocampus of the rats was fixed in 10% formalin (the control was cerebral cortex of a known case of AD). Paraffin sections of about 8 μm were prepared.

Bielschowsky staining relies on sensitizing the nerve fibers with 20% silver nitrate reagent and treatment with ammoniacal silver.

Gene expression analysis

Upon completion of the treatment (day 28), the animals were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (10 mg/kg) solutions. Blood sample was taken from cardiac ventricles using a 5 mL syringe in both ethylenediaminetetraacetic acid-containing and clotting tubes. Blood samples were allowed to clot for 30 min at room temperature and centrifuged at 2000 rpm for 10 min to isolate the serum. Using the enzyme-linked immunosorbent assay (ELISA) kit (Abcam), the expression patterns of the *IL-4* and *IL-6* cytokines were detected in the serum according to the manufacturer's instructions.

The RNA of each rat was extracted from the peripheral blood lymphocytes using RNX-Plus™ kit (Cinnagen, Tehran, I.R. Iran) according to the manufacturer's protocol. RNA concentration, purity, and integrity were checked using a Nanospec cube biophotometer and agarose gel electrophoresis. cDNA synthesis was completed using RevertAid™ first strand cDNA synthesis kit (Fermentas) conferring to the manufacturer's protocol. The relative expression level of the *CDK11-P58* gene was measured by a real-time PCR assessment conducted via SYBR Premix Ex Taq™ II kit (TaKaRa). Primers were designed using Allele ID 7.0 *CDK11P58* F: GGACTATGTGCCCGACTCTC, R: GGTGCCTTCTTCAATCCTGTTC. *GAPDH* F: AGGGCTGCCTTCTCTTGTGAC, R: TGGGTAGAAT CATACTGGAACATGT. The *GAPDH* gene was used as an endogenous control and normalization of the expression of the target genes. Cycle threshold (Ct) values were used to calculate fold changes in gene expression using $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were analyzed by SPSS 22 (IBM, USA) through one-way ANOVA followed by *post hoc* test. All values were expressed as mean ± standard error of mean (SEM). Statistical significance was accepted at $P < 0.05$. The relative gene expression analysis was performed by REST© software.

Results

Behavioral outcomes

The MWM test data displayed significantly increased time of finding the hidden platform in the AD group in comparison with the other groups. The treatment groups showed an improved operation when compared with the AD group ($P < 0.001$). The group treated with Hyo showeda significant change in platform finding and swimming speed when compared with the AD group ($P < 0.01$). Furthermore, Hyo treated rats had better functionality in comparison with Dz -treated rats [Figure 1]. The results of positive control group were not significantly different
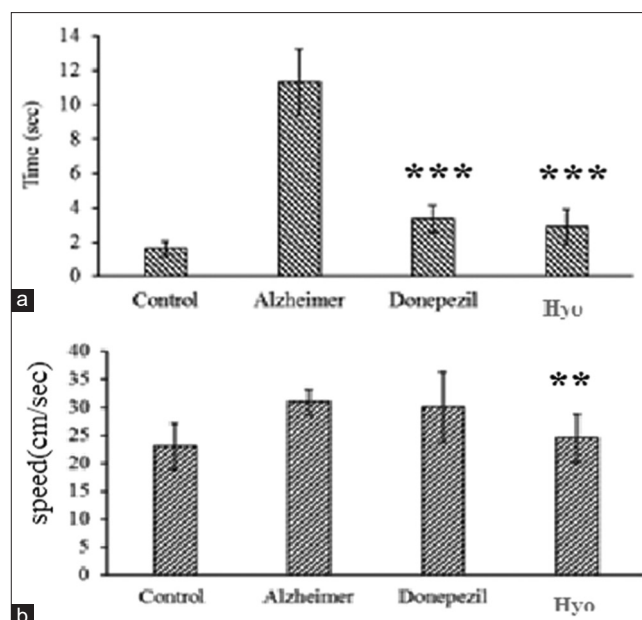

Figure 1: Morris water maze results. (a) Time; and (b) speed. a: ***Indicates significant decreased time of finding the hidden platform in rats that were treated with donepezil and hyoscyamoside (Hyo) when compared with Alzheimer's disease rats ($P < 0.001$). **Shows significant change in speed of swimming in rats that were treated with Hyo when compared with AD ($P < 0.01$)

from the negative group. The negative control group was expressed in the statistical results.

Cytokines and gene expression

The expression analysis of the cytokines by ELISA assay uncovered significantly decreased expression level of serum *IL-6* in rats that received Dz (110.08 ± 4.48) and Hyo in comparison with AD rats ($P < 0.001$) [Figure 2a and b]. Moreover, *IL-4* serum level was significantly increased in the rats that were treated by Dz (84.79 ± 3.16) and Hyo (94.48 ± 3,73) compared with the AD rats ($P < 0.001$) [Figure 2].

Evaluation of the expression level of RNA of the *CDK11P58* gene extracted from peripheral lymphocytes by RT-PCR showed that the *CDK11P58* had significantly ($P < 0.001$) decreased expression level in Dz (1.049 ± 0.10) and Hyo treatment groups when compared with the AD rats [Figure 3].

Histological findings

Bielschowsky staining was applied to evaluate plaque aggregation in the cerebral hippocampus and inflammation. There was neither plaque nor leukocyte in the control group as expected [Figure 4a]. While microscopic histopathological analysis revealed aggregation of both senile plaques and leukocytes in AD rats that did not receive any treatment. Red neuron formation due to neurotoxicity effect of Aβ was obvious [Figure 4b]. The histopathological observations revealed that the plaque formation was reduced in the treatment groups effectively [Figure 4c and d].
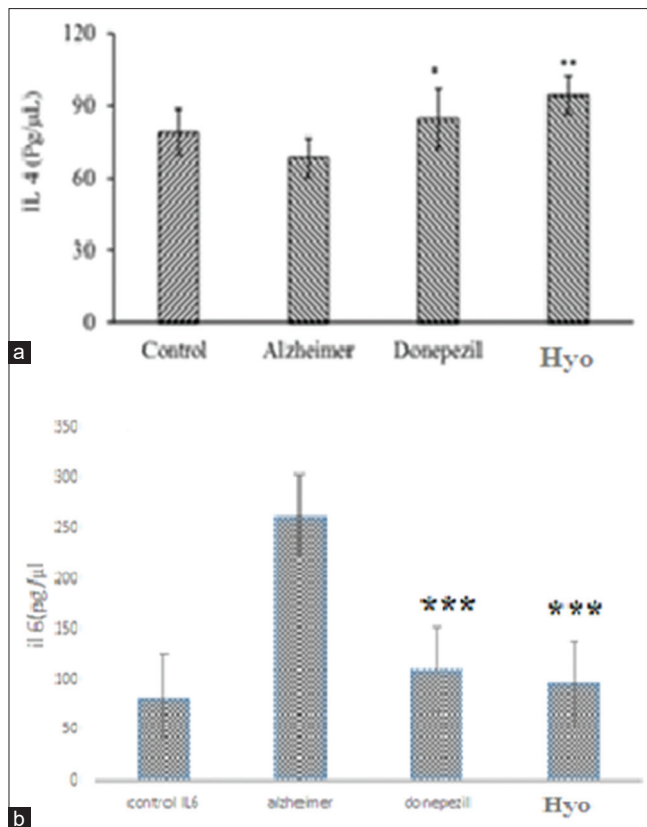
Figure 2: Serum level of, interleukin; a, *IL-4*; and b, *IL-6* were evaluated by ELISA assay. *,***Indicate signifiant difference (*P*<0.001, respectively) in comparison with Alzheimer's disease group but please change in figuer
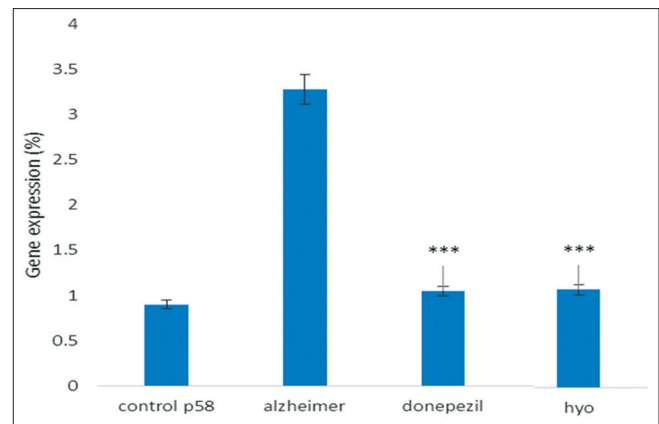

Figure 3: *CDK11-P58* expression levels were examined by real-time polymerase chain reactions. ***Indicates significant differences (*P* < 0.001) compared with Alzheimer's disease group

Discussion

AD accounts for the most common cause of age-dependent dementia. Aggregation of Aβ plaques in hippocampus is considered as the trigger for downstream pathophysiological events including formation and accumulation of neurofibrillary tangles, neuroinflammation, and disturbance of cholinergic system. Here, we investigated the preventive effect of Dz and Hyo on inhibiting the senile plaque formation and relieving the neuroinflammation.

In this study, we could establish Bielschowsky staining for monitoring the effect of Aβ on the hippocampus of the rats. The Aβ aggregation in the brain in our study is in concordance with the previous studies as the most well-established pathophysiological mechanism of the disease. Our observation of the leukocytes in the hippocampus could be because the cytokines called white blood cells to the position for neuroinflammation. The cascade of such biological events leads to memory loss and cognitive impairment, compatible with our MWM findings. The extended time of finding the platform by the AD rats confirmed that aggregation of Aβ plaques is pathologic for learning, memory, and cognition.

In the present study, we observed reduced formation of Aβ in the hippocampus of AD-induced rats that were treated by Dz and Hyo along with improved brain function.

In parallel, previous studies brought enough evidence that plant-derived steroidal saponin might slow down the progression of neurocognitive manifestations of AD.[17] Dz enabled the rat septal neuronal cells to resist the harmful effects of β-amyloid[19] of AChEIs and the inhibition of the production of inflammatory substances from specialized cells. It has been proved that *in vivo* management with AChEI downregulates the expression and production of cytokines (*IL-1β*, *IL-6*, and *TNFα*) and upregulates the expression and production of *IL-4* in peripheral blood cells of Alzheimer's patients.[19]

Treatment with diosgenin significantly decreased the amount of Aβ-stained plaques and other dead cells in the granule cell layer of the dentate gyrus. Inhibition of AChE activity and Bax/Bcl-2 expression.[20] Steroidal saponins inhibit the generation of nitric oxide and ROS, expression of iNOS, *IL-6*, and *IL-1β*, and anti-inflammatory activities.[21]

In addition, it has been demonstrated that *IL-6* is present in the early stages of plaque formation.[21] Our results indicate the downregulation of *IL-6* after treatment and decreased senile plaques when compared with AD rats. It is concluded that these treatments have an influence on declining the inflammation in the injury sites of the brain. Overexpression of *IL-4* in the treatment group is in concordance with our histopathological and behavioral findings. As *IL-4* is an anti-inflammatory cytokine, it might have enhanced agents that reduce immune response. Studies have shown that cholinesterase therapy in AD patients leads to increased level of *IL-4* which might be an evidence for modifying neuroinflammation.[22] Oligomeric Aβ pushes the neurons into cell cycle which leads to neurodegeneration and cell death.[23] The *CDK11* genes has been observed to express in inflammation, cytoplasm, and cellular process in neurons of AD patients while in healthy cases it is expressed in the nuclei of post-mitotic neurons.[23] Stimulation of cells in central nervous system inflammation primes to a disturbance of crosstalk among
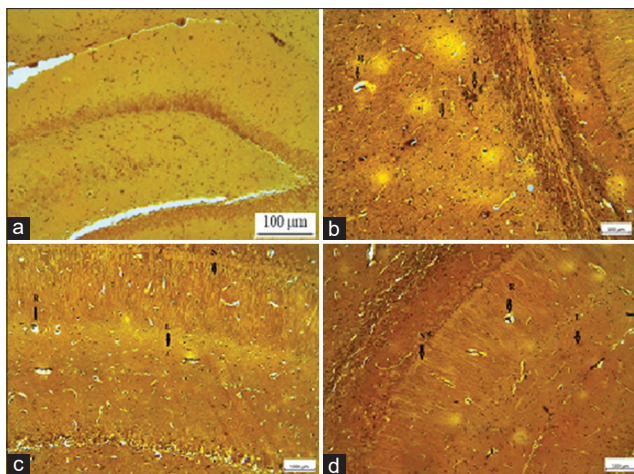
Figure 4: Bielschowsky staining of the hippocampus (×400). (a) Normal tissue of the hippocampus showed no injury; (b) (Alzheimer's disease group): B is indicating R: Red neurons due to neuronal injury. Red neurons form as a consequence of plaque aggregation. L: leukocytes accumulated in the hippocampus. P: Plaque amyloid beta. (c) (treated with donepezil): N: is demonstrating healthy neurons, (d) (treated with hyoscyamoside)

astrocytes and neurons, and that this could be attributed to the loss of neurons. The *CDK11-p58* overexpression leads to Tau hyperphosphorylation with its kinase activity causing neurotoxicity in AD patients. Given Aβ to M17 cell line leads to increased expression of *CDK11-p58*.[7] As observed in our experiments, *CDK11-p58* gene, *was* elevated in AD rats after Aβ induction. Expression levels were decreased in treatment groups which signify the effect of our treatments on senile plaque aggregation, which is compatible with our histopathological findings. So, control of inflammation and oxidative stress processes in a simultaneous way can discontinue pathological occurrences related to AD.

Conclusions

Overall, Dz and Hyo exhibited a positive effect on brain cognitive function, MWM test, inhibition of plaque formation, and modulation of neuroinflammation by targeting inflammatory cytokines and involved gene expression. As long-term Dz consumption is accompanied with adverse effects searching through herbal therapeutic components might lead to the introduction of novel alternative medications with lower side effects and cost. Hyo, as a natural product with antioxidant capacity, could present a preventive effect. However, more investigations such as exact extraction methods seem to be necessary for validation of its efficiency for future therapies.

It suggests that Hyo could be used as a nutritional supplement in familial AD to prevent plaque formation and inflammation. It hopes to find a new way to treat and improve Alzheimer's. Indeed, more investigations are required to introduce these components as probable diagnostic, prognostic, and therapeutic biomarkers in the future.

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