

The inhibitory effects of bile acids on catalytic and non-catalytic functions of acetylcholinesterase as a therapeutic target in Alzheimer's disease

Leila Sadeghi*, Reza Yekta, Gholamreza Dehghan

Department of Animal Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran,

* Email: l.sadeghi@tabrizu.ac.ir and l.sadeghi66@yahoo.com

Acetylcholine is a fast-acting neurotransmitter in synapses and neuromuscular junctions that is decreased in Alzheimer's disease (AD) by hyper-activation of acetylcholinesterase (AChE), which leads to progressive loss of memory and neurobehavioral abnormalities. Therefore, AChE inhibitors have therapeutic potential in AD that could include natural compounds such as bile acids. Bile acids, as potent molecules, could improve some types of neurodegenerative diseases via antioxidant effects and other unknown mechanisms. The aim of this study was to investigate beneficial effects of bile acids on AChE catalytic and non-catalytic functions, amyloid plaque deposit and memory in a rat model of AD. The effects of sodium deoxycholate and cholic acid on AChE activity were assessed by *in vitro* assay. Then, the bile acids' potential therapeutic effects were investigated on nucleus basalis of Meynert lesioned rats using behavioral evaluation, biochemical tests and histological methods. Molecular docking simulation was also implemented to investigate the possible interaction between bile acids and AChE. According to the *in vitro* and *in vivo* results, sodium deoxycholate could efficiently inhibit the catalytic function of the enzyme by interacting with the catalytic site, while cholic acid interacted with the peripheral anionic site and inhibited chaperone activity of the enzyme that led to the reduced amyloid plaque deposition. The co-administration of cholic acid and sodium deoxycholate showed these compounds are able to simultaneously inhibit the catalytic and non-catalytic functions of the enzyme. This study clarifies the roles of natural bile acids in the nervous system and in AChE function through multiple experimental and simulation methods.

Key words: neuronal impairment, sodium deoxycholate, cholic acid, amyloid plaque deposit, hippocampus, acetylcholinesterase

INTRODUCTION

Alzheimer's disease (AD) is a progressive dementia, hallmarked by amyloid plaque deposition and characterized by memory loss and cognitive dysfunction (Dong et al., 2012). Cumulative evidence strongly supports that decreased acetylcholine (ACh) neurotransmitter, as a result of acetylcholinesterase (AChE) hyperactivation, plays an important role in the occurrence and development of neurological disorders related to AD in patients and animal models (Anand and Singh, 2013). Therefore, AChE inhibition by various drugs, such as donepezil, rivastigmine and galantamine, are used to improve symptoms of AD pathophysiology (Anand and Singh, 2013).

AChE which belongs to the α/β hydrolase fold family is an ACh-hydrolyzing enzyme in the synapse and neuromuscular junction that terminates cholinergic impulses in nerves (Holmquist, 2000). Despite the catalytic activity, AChE participates in multiple biological functions, such as neuronal cell differentiation, dendrite and axon formation, cell adhesion and neurodegeneration in Alzheimer's disease (Dong et al., 2004). Two sites of ligand interaction contribute to the catalytic and non-catalytic functions of AChE: a catalytic active site (CAS) at the bottom of a deep and narrow gorge and a peripheral anionic site (PAS) that is surrounded by negatively charged and hydrophobic residues (Johnson and Moore, 2006). Previous studies confirmed that AChE plays an

effective role in A β plaque deposition by mediating PAS chaperone activity (García-Ayllón, 2011). Therefore, natural and synthetic compounds that effectively bind to the active sites, CAS and PAS, could potentially inhibit ACh hydrolysis and amyloid plaque formation (Holmquist, 2000; Liu et al., 2013).

It was also previously reported that endogenous bile acids are potent anti-apoptotic agents in neuronal cells that are exposed to A β (Viana et al., 2010). They are further known as powerful neuroprotective agents in various neurodegenerative diseases. Bile acids are structurally known as a group of antioxidants that are derived from cholesterol. This group can pass through the blood brain barrier (BBB) and was also shown to protect neuronal cells against oxidative damage (Lo et al., 2013). The neuroprotective mechanisms of bile acids are not completely understood but prior studies suggest that bile acid derivatives could potentially block the apoptosis process through stabilization of the mitochondrial membrane potential ($\Delta\Psi_m$) and regulation of gene expression (Viana et al., 2010; Ackerman and Gerhard, 2016). Therefore, the main goal of this study was to evaluate the effects of cholic acid (CA) and sodium deoxycholate (SDC), as main bile acids, on the catalytic and non-catalytic functions of AChE. The principal binding site of bile acids on AChE were investigated by molecular docking analysis. Their potential therapeutic effects were investigated on nucleus basalis of Meynert lesioned (NBML) rats, a widely accepted model of AD that manifests pathophysiological and behavioral signs of the disease (Toledano and Alvarez, 2004; Gratwicke et al., 2013). Previous results demonstrated that impaired memory related to AD is dependent on hippocampal function (Moodley et al., 2015; Sadeghi et al., 2018). Therefore, we investigated CD and/or SDC for beneficial effects on the NBML rats' hippocampus through biochemical analysis, immunological evaluation and histological studies.

METHODS

Animals

In vivo study was conducted on experimental animals by using 4.5–6 months adult male Wistar rats, weighing 250–300 g obtained from the animal house. All tests were carried out at 20–25°C (12 hours light and 12 hours dark). Municipal tap water was used as drinking water and compressed food as nutrition (consisting of 23% protein, 3.0% crude fat, 7.0% crude fiber: 8% acid insoluble ash, 1–2.5% calcium, 0.9% phosphorus, 0.5–1% sodium, 12% moisture). All the experimental works were approved by the Ethical Committee of Tabriz Uni-

versity of Medical Science (Tabriz, Iran) and conform to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

AChE activity assessment in hippocampal primary culture homogenate

Primary neuronal cultures of rat hippocampus were prepared according to a previous study with some modifications (Seibenhener and Wooten, 2012). Dissected hippocampus (isolated from neonatal rat) was triturated by trypsin and mechanical disruption. The cells were differentiated in neurobasal medium supplemented with B27 and FGF2 for 7 days. The resulting cells were homogenized in cold buffer phosphate, 28 mM (pH 7.8), containing Triton X100 (Sadeghi et al., 2018). The homogenized samples were then centrifuged at 13,000 g for 20 min and subsequent supernatants were considered for AChE assay. AChE activity was determined based on spectroscopic monitoring of 5-thio-2-nitrobenzoate anion production at 430 nm during the reaction between 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and thiocholine. In this reaction, acetylcholine iodide as a substrate was converted to thiocholine that reacts with DTNB and produces 5-thio-2-nitrobenzoate. The final concentrations of DTNB and substrate were 0.33 mM and 1.56 mM, respectively, in all assay medium. The activity of the enzyme was expressed as μ mole of ACh hydrolyzed per minute per milligram protein in the absence and presence of bile acids (10 μ M), individually. The homogenate's protein concentration was evaluated according to the Bradford method (Bradford, 1976). All assays were done in five replicates.

Molecular docking analysis

In order to precisely assess the affinity of CA and SDC to AChE, Auto Dock software (version 4.2) was used to simulate the binding process of bile acid molecules to AChE enzyme (Morris et al., 2009). For this aim, firstly, the crystal structure of AChE (PDB ID: 1DX6) was taken from the Protein Data Bank (<https://www.rcsb.org/>). Since different subunits of the enzyme are the same, the subunit A was selected to perform docking analysis precisely. The 3D molecular structures of CA and SDC were obtained from the PubChem website (<https://pubchem.ncbi.nlm.nih.gov>). Their structural conformations were minimized by Gaussian 03 programs based on a theoretical level of B3LYP with 6–31G basis set. Polar hydrogen atoms were added into AChE as a target and Kollman charges were also calculated. In addition, the rotation of bile acid molecules was defined and rotatable bonds were detected. To

identify the ideal binding site of each bile acid on AChE protein, a docking calculation using the Lamarckian genetic algorithm (LGA) search method was performed. All other calculation parameters were default settings. Chimera 1.10.1 program was used to visualize the obtained results (Rizvi et al., 2013).

***In vivo* experimental design**

NBML rats were used as an AD model as follows: rats were anesthetized with ketamine (125 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and then placed in a Stoelting stereotaxic apparatus. The midline of clean scalp was notched and a burr hole was drilled into the skull. Following that, ibotenic acid was injected at the NBM nucleus with coordination as follows: AP=1.2, ML=±3.2, and DV=7.5 mm from the surface of skull (Paxinos and Watson, 2005). Each side of the NBM was injected with 1 µl of 5 µg/µl ibotenic acid solution with a microinjection pump at the speed of 120 µ lit/h. After 7 days of recovery, the animals were divided randomly into three groups as follows:

Control: rats were submitted to surgery and injected with 1 µl saline (as equivalent shock obtained by surgery and local injection) and were orally administered saline after recovery for 25 days.

Rat model of AD: NBML rats in which both NBM nuclei were bilaterally destroyed by ibotenic acid during surgery. These rats received saline orally after recovery for 25 days.

NBML rats treated with bile acids: this group consisted of 24 rats whose NBM nuclei were bilaterally destroyed by ibotenic acid. After recovery, the AD-like rats were randomly divided into three groups (8 rats in each group) and two groups were treated orally with 30 mg/kg of SDC or CA for 25 days. The third group received 15 mg/kg SDC+15 mg/kg CA to study synergic effects of both bile acids. The dose of bile acids and the experimental time duration were selected according to a previous study (Lo et al., 2013).

To confirm the increase of bile acid levels in the brain tissue of rats after 25 days of treatment (15 mg/kg SDC+15 mg/kg CA), the concentration of CA and SDC were evaluated in brain tissue by using CA and DCA ELISA Kit (Cell BIOLABS and MYBIOSOURCE). According to the obtained results, CA and DCA concentrations in the control group were calculated at 0.1 ± 0.02 nmol/g and 0.06 ± 0.01 nmol/g, respectively, which increased to 0.24 ± 0.08 nmol/g and 0.18 ± 0.03 nmol/g, respectively, in rats that received combined bile acids during the 25 days. Therefore, oral administration of bile acids enhanced these molecules' concentration more than two-fold in brain tissue.

Western blotting analysis

Western blotting was carried out according to a standard protocol (Sadeghi et al., 2017). After SDS-PAGE, the proteins were transferred onto PVDF membrane under 140 V for 1.5-2 h in transfer buffer. The membrane was probed with the primary [Anti-Glial Fibrillary Acidic Protein (GFAP) antibody (G3893, Sigma-Aldrich), Anti-beta Amyloid 1-42 antibody (ab10148, Abcam), rat specific anti-acetylcholinesterase antibody (ab31276, Abcam), rat specific anti-amyloid precursor protein (APP) antibody (ab2072, Abcam)] and secondary specific antibodies after blocking and washed four times in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05 % Tween 20). An ECL detection kit was used to visualize bands containing specific proteins according to the manual. Anti β-actin (1:1000) (Cell Signaling Technology) was used as a housekeeping protein to control protein concentration.

Histological studies

For histological analysis, the rats from each experimental group were sacrificed under deep ketamine and xylazine anesthesia and before fixation with 4% formaldehyde and were subsequently perfused with 0.3% sodium sulphide in 0.1 M phosphate buffer. The separated brains were stored in 30% sucrose for cryoprotection and sectioned on a freezing microtome at 8 µm. The tissue sections were stained with Thioflavin T, a general marker of amyloid deposits, and studied by a fluorescent microscope. To this purpose, the tissue sections were incubated with freshly filtered 0.05 % Thioflavin T solution in PBS for 8 min at room temperature (Batelli et al., 2008).

Statistical analysis

SPSS10 software was used to analyze the data. Analysis and identification of the differences between experimental groups and control were performed using the t-test and $p<0.05$ was considered as a significant difference level.

RESULTS

Bile acids bind to the active site gorge and inhibit AChE catalytic activity

AChE belongs to the serine hydrolase family that contains the α/β hydrolase fold as a common structural element (Holmquist, 2000). A hallmark of AChE

is its catalytic activity that can be assessed by the Ellman method, a sensitive, rapid and reliable method for detecting very low amounts of AChE catalytic function (Sadeghi et al., 2018). In the present study, primary hippocampal neuron cultures were used as a source of AChE to investigate the possible effects of bile acids (CA, SDC and CA+SDC) on catalytic activity of AChE. The results showed that bile acids could significantly inhibit hydrolyzing activity of the enzyme. The catalytic function of AChE was reduced from 6.37 ± 0.52 to 5.56 ± 0.31 $\mu\text{mol}/\text{min} \cdot \text{mg}$ (protein) in the presence of CA (10 μM), while SDC reduced AChE activity to 3.64 ± 0.37 $\mu\text{mol}/\text{min} \cdot \text{mg}$ (protein). Further, it was shown that 10 μM of combined bile acids (5 μM CA+ 5 μM SDC) could reduce the enzyme activity to 2.07 ± 0.23 $\mu\text{mol}/\text{min} \cdot \text{mg}$ (protein). The maximum rates of AChE inhibition occurred in the presence of SDC+CA and decreased more than three-fold (Table I).

Molecular docking simulation

Molecular docking simulation revealed that both of the evaluated bile acids were capable of binding to the active site gorge of AChE at different affinities. The binding energy was calculated as -7.76 and -8.94, respectively, for the binding of CA and SDC to the active site gorge. It was shown that the active site of AChE consists of catalytic residues (Ser 203, His 447 and Glu 334), which are located within the gorge, similar to other AChEs in mammals (Johnson and Moore, 2006; García-Ayllón et al., 2011; Liu et al., 2017). Also, some anionic and hydrophobic amino acids surround the entrance of the active site known as the PAS site (Tyr 286, Trp 286, Phe 338, Val 294 and Leu 289) (Johnson and Moore, 2006; García-Ayllón et al., 2011). Fig. 1 shows the binding sites of bile acids on the active site of AChE. It was indicated that CA due to the bulky structure could bind to PAS and is not able to reach to the catalytic site at deep of the gorge so could limit substrate access to the catalytic site. While SDC could pass through the PAS

tunnel and reach to CAS which limits substrate binding to the active site. All docking results are listed in Table I.

Bile acids treatment attenuated catalytic activity of hippocampal AChE that was increased by NBM lesion

NBM lesion causes impaired spatial navigation according to Fig. S1 (supplementary data) in experimental animals. The pathogenesis of AD has been linked to AChE activation that is also accompanied by β -amyloid deposition (Dong et al., 2004; Johnson and Moore, 2006). Therefore, to investigate whether NBM lesion and oral administration of bile acids could affect AChE activity in the rat hippocampus, the enzyme activity in homogenate tissue was evaluated for all groups. In agreement with our previous study (Sadeghi et al., 2013), the results showed that AChE activity was significantly increased in NBML rats up to 2982.71 ± 152.26 $\text{nmol}/\text{min} \cdot \text{mg}$ protein, while the enzyme activity in the control hippocampus was calculated to be 525.34 ± 34.52 $\text{nmol}/\text{min} \cdot \text{mg}$ protein (Fig. 2). NBML rats that treated with 30 mg/kg of SDC and/or CA for 25 days, showed reduced AChE activity compared with the NBML rats that treated with saline. Fig. 2 shows that the activity of AChE was remarkably reduced (3-fold) in NBM lesioned rats were administered 30 mg/kg of SDC. NBML rats received 30 mg/kg of CA revealed a two-fold reduction in AChE activity in hippocampal tissue. The enzyme activity was, however, remarkably inhibited in the rats that received both of the bile acids (520.13 ± 50.25 $\text{nmol}/\text{min} \cdot \text{mg}$ protein), indicating synergistic effects.

Bile acids improved abnormal expression of GFAP, A β and AChE

Western blot analysis confirmed the increased expression of GFAP, a well-known biomarker for neuronal

Table I. The effects of bile acids on hydrolytic activity of AChE and binding parameters of each bile acid on the enzyme. Experimental data was expressed as the mean \pm S.E.M. of at least five independent tests.

Bile acids	AChE activity ($\mu\text{mol}/\text{min} \cdot \text{mg}$ protein)	Binding energy (kcal/mol)	Interacting amino acids
Control	6.37 ± 0.52	-	-
Cholic acid (CA)	5.56 ± 0.3	-7.76	Ser 293, Arg 296, Tyr 124, Tyr 341, Val 294, Leu 289, Trp 286, Phe 297, Tyr 337, Phe 338
Sodium deoxycholate (SDC)	3.64 ± 0.37	-8.94	Tyr 133, Asn 87, Pro 88, Glu 202, Trp 86, Tyr 124, Tyr 337 and His 447
CA+SDC	2.07 ± 0.23	-	-

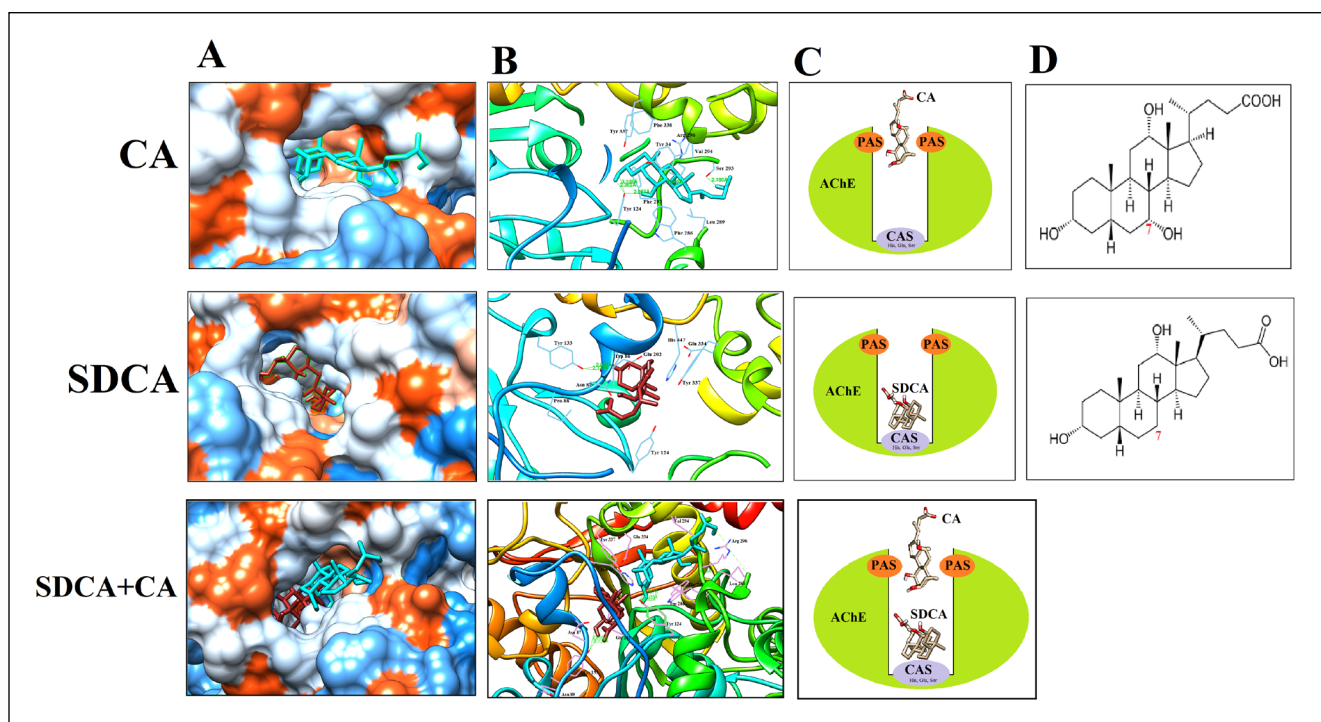


Fig. 1. Bile acid molecules in the AChE active site gorge. (A) shows the surface view of the active site cavity representing the crystal structure of AChE with two bile acids bound. (B) shows a cartoon form of one subunit of AChE that binds to bile acids by hydrophobic interactions and hydrogen bonds. CA and SDC are represented as a cyan and brown molecule, respectively. (C) indicates the binding position of each bile acid in the active site gorge in comparison with PAS and CAS sites. (D) shows molecular structures of each bile acid with the C₇ marked in red.

damage in ibotenic acid received hippocampus (Lei et al., 2015). In this study, it was revealed that the GFAP content of hippocampal tissue was reduced in the rats treated with bile acids, especially the rats that received CA+SDC daily. Our results also revealed that AChE expression was enhanced in hippocampal tissue of NBML rats, while bile acids significantly reduced its expression. Fig. 3 reveals that the effects of CA and the combination of both bile acids are more significant than SDC treatment. According to the results, the level of A β content was six-fold in the NBML rats compared with the control. Interestingly, CA caused a greater decrease in the level of A β in hippocampal cells (more than five-fold) than SDC (near to two-fold). Our results also confirmed nonlinear cumulative effects of CA and SDC in the reduction of AChE expression.

Bile acids improved histopathological abnormalities induced by NBM lesion

In this investigation, histological studies were done as confirmation of biochemical results. Thioflavin staining demonstrated a clear abundance of amyloid plaques in the hippocampal tissue of NBML rats; however, deposits were absent in the control rats. In agree-

ment with a previous study (Lo et al., 2013), thioflavin plaque numbers were reduced in the hippocampus of bile acids-supplemented-NBML rat compared to the NBML rats that treated with saline. Thioflavin plaque

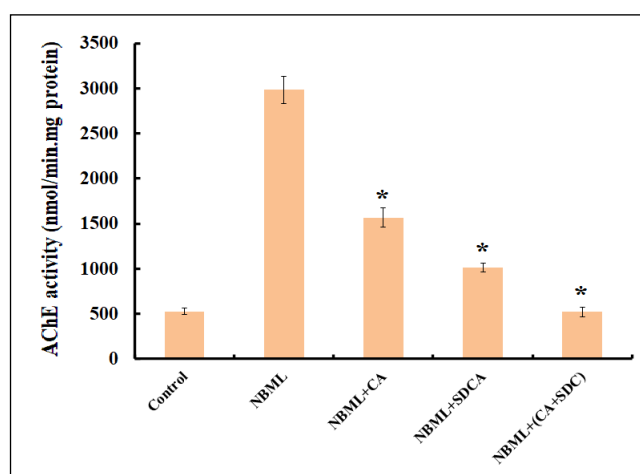


Fig. 2. AChE activity in the hippocampus tissue. Results showed a significant increase in hydrolyzing activity in NBML rat's hippocampus that was attenuated with bile acids treatment. Co-administration of CA and SDC was most significant. The data were expressed as mean \pm S.E.M. of five independent experiments and an asterisk (*) symbol indicates significant changes in comparison with NBML rats ($p < 0.05$).

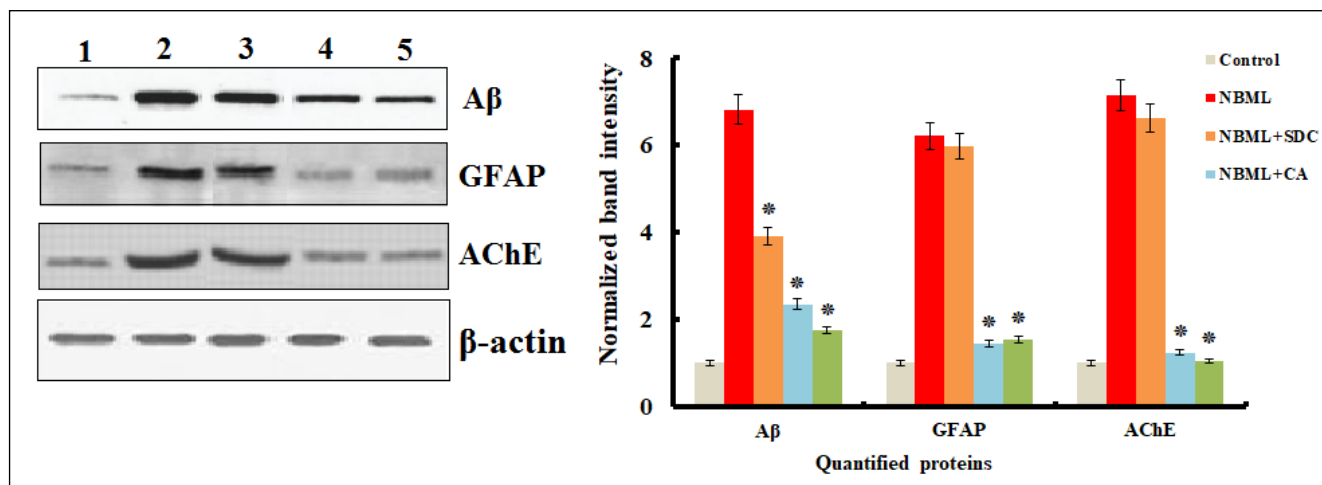


Fig. 3. Immunoblotting studies. Results showed upregulation of AChE, A β and GFAP proteins in NBML rats that can be interpreted as hippocampal damage, which improved with oral administration of bile acids. (A) Lane 1 represents control hippocampus, lane 2 shows results in NBML rats. Lane 3 represents SDC-treated NBML rats, lane 4 represents NBML rats treated with CA and lane 5 shows results in NBML rats that received CA+SDC. (B) Immunoblotting results quantified with Image J software as a plot. The data were expressed as mean \pm S.E.M. of at least three independent experiments and asterisk (*) symbol indicates significant changes in comparison with NBML rats ($p < 0.05$).

numbers significantly decreased in the groups treated with SDC and CA. Fig. 4 also shows cumulative effects of both bile acids in AD rats that received SDC and CA simultaneously and resulted to decreased number and size of plaques.

DISCUSSION

We investigated the effects of bile acids (SDC and CA) on hippocampal AChE activity as an important target in AD therapy. The *in vitro* investigation of AChE was carried out using Ellman's method with crude enzyme that was extracted from primary neuronal cultures approximating neonatal rat hippocampus (Sadeghi et al., 2018). The results showed that both bile acids could significantly inhibit AChE hydrolytic activity (Table I) possibly due to substrate accessibility restriction. AChE protein possesses two sites for ligand interactions: an active (hydrolytic) site within a deep narrow gorge (CAS), including Ser203, Glu334 and His447, and a peripheral anionic site (PAS) that consists of a group of negatively charged and hydrophobic residues surrounding the entrance site (Prasher, 2004; Liu et al., 2017). The CAS is located within the inner site of the enzyme and substrate molecules must pass through the PAS tunnel to reach to the CAS, the distance between the two position estimated to be approximately 12 Å. Molecular docking studies revealed that SDC, upon binding to the CAS, could inhibit esterase activity more significantly; however, since CA could not reach to the catalytic site within the gorge,

it could not effectively inhibit hydrolytic activity but could possibly limit substrate access by binding to the PAS site. Structural and docking analysis of bile acids revealed that the presence of hydroxyl group at C₇ of the sterol rings limited bile acid from penetrating to the active site gorge possibly due to the increasing molecular dimensions that lead to decreased flexibility. Interestingly, AChE activity decreased more significantly in the presence of an equivalent combination of CA and SDC, which suggests synergistic inhibitory effects. Regarding the different physiological roles of PAS and CAS in biological systems, the effects of CA (binds to PAS), SDC (binds to CAS) and CA+SDC (bind to PAS and CAS) on biochemical and molecular aspects of AD were investigated by using NBML rats, a recognized animal model of AD.

Local injection of ibotenic acid damaged acetylcholine manufacturing cells in the NBM and caused AChE hyper-activation in hippocampus that ultimately led to AD pathophysiological signs (Wenk, 1990; Anand and Singh, 2013). According to the results, oral administration of bile acids for 25 days enhanced CA and DCA concentrations in the brain tissue more than two-fold. Therefore, similar to the *in vitro* assay, oral administration of SDC and CA effectively decreased the activity of hippocampal AChE. NBML rats that received both of bile acids in similar doses (15 mg/kg CA+15 mg/kg SDC) exhibited minimal hippocampal AChE hydrolytic activity (near control values), which indicates non-linear cumulative effects similar to the *in vitro* results. Immunoblotting analysis also confirmed enhanced AChE expression, due to NBM lesion,

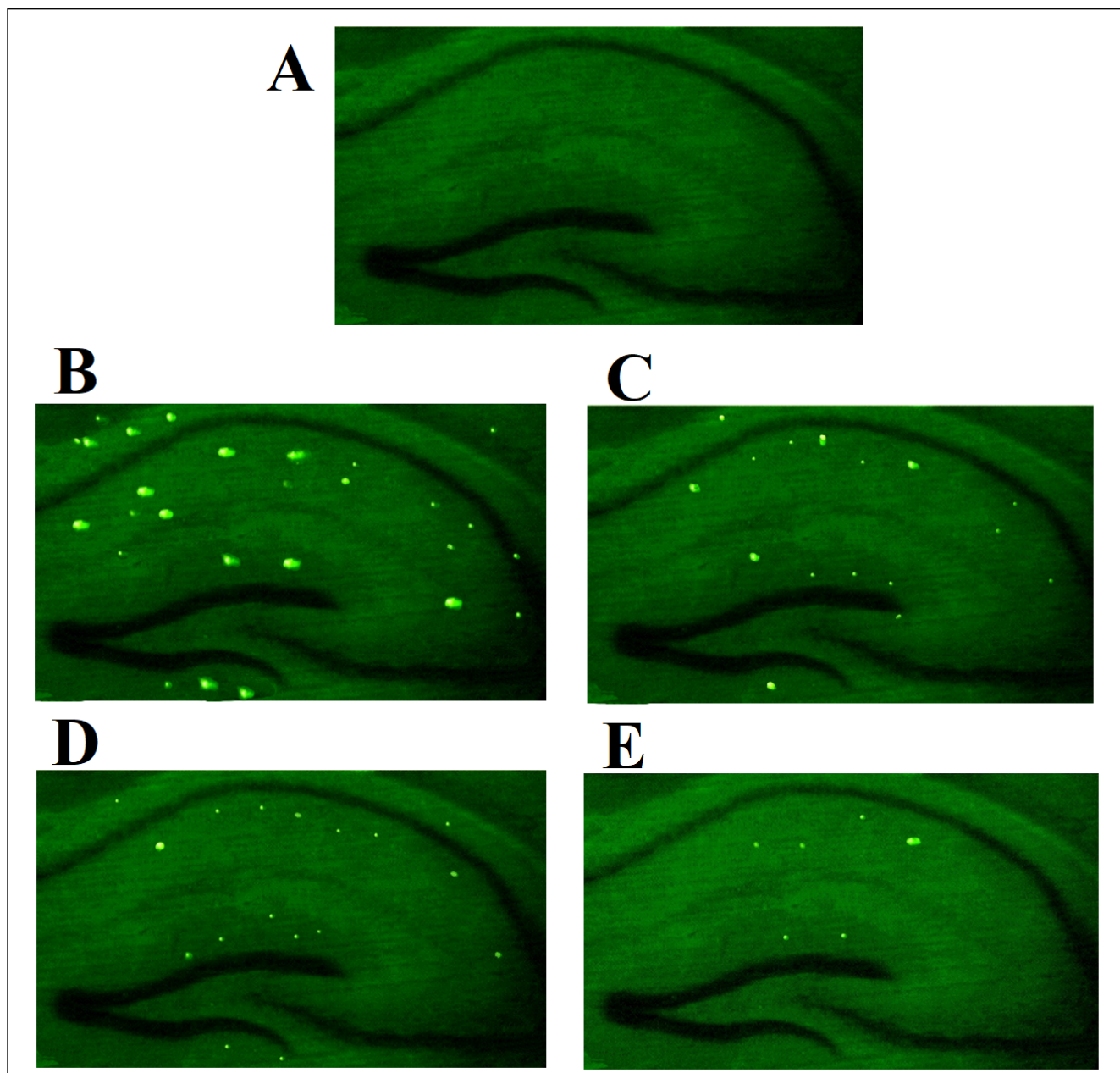


Fig. 4. Thioflavin staining of hippocampus tissue. Results showed absence of amyloid plaques in control rats. Hippocampal sections representing NBML rats showed high amounts of deposited amyloids. Amyloid deposit amounts were reduced in rats that received the PAS-interacting bile acid (CA) versus SDC, which could not bind to the PAS.

decreased significantly with bile acids treatment, especially in rats that received CA and CA+SDC. NBM lesion caused an approximately 4-fold increase in AChE expression but its activity increased more than 5.6-fold. Therefore, activation and increased expression of AChE were taking place simultaneously. Previous experiments indicate that bile acids could regulate gene expression through binding to the G-protein-coupled bile acid membrane receptor 1 (TGR5) and bile acid nuclear Farnesoid X receptor (FXR) at

the membrane and inside the nucleus, respectively (Mertens et al., 2017). SDC treatment reduced AChE expression slightly but decreased its catalytic activity more than 4-fold. The reduction of AChE expression and catalytic activity in the presence of CA seems to be equal; therefore, it appears that CA reduces AChE's hydrolyzing activity primarily through regulation of gene expression. Therefore, these results reveal that the expression and activity of AChE enzyme could be regulated by different pathways.

AChE is colocalized with A β in pre-amyloid deposits, mature senile plaques and cerebral blood vessels in the brain of AD patients, which suggests its role in promotion and development of A β plaques (Inestrosa et al., 1996). *In vitro* and *in silico* analyses confirmed that A β has a marked tendency to interact with the PAS site mainly via hydrophobic interactions (García-Ayllón et al., 2011). The increased activity and expression of AChE in NBML rats suggests that the chaperone role of AChE also improved, which is associated with the PAS site and is responsible for A β polymerization. Interestingly, it was observed that CA binds to the PAS site and reduces amyloid plaques more significantly than SDC, which mainly interacts with CAS. It is possible that CA could have bound to PAS, inhibited the chaperone function of AChE and attenuated A β plaques deposition, leading to the decrease of plaque number and size. NBM lesion by ibotenic acid caused cholinergic system impairment and led to the increased GFAP content in hippocampal cells that confirmed AD is a neurodegenerative disease (Moodley et al., 2015; Lei et al., 2015). The treatment of rats with CA or CA+SDC resulted in a strong decrease in neuronal damage compared to rats that received SDC. Simultaneous AChE upregulation and increased expression of A β and GFAP suggests a possible role for AChE in the regulation of the genes involved in apoptosis as a non-catalytic function (Dong et al., 2004). The effect of bile acids at the molecular level resulted in the refinement of behavioral abnormalities caused by NBM lesion in rats. Fig. S1 reveals that NBML rats treated with CA+SDC for 25 days took less time to reach the platform than rats that received normal saline, SDC or CA.

Overall, this is an original report of the bile acids' therapeutic effects on NBM lesion-induced pathology through *in vivo*, *in vitro* and *in silico* experiments, which results suggests a novel approach in AD therapy. Our results confirmed that SDC more strongly inhibited hydrolytic functions, possibly by binding to the catalytic site, while CA binds to the PAS site and inhibited non-catalytic functions of AChE such as plaque formation and gene regulation. Therefore, in the presence of both bile acids, CAS and PAS were inhibited simultaneously and both of the catalytic and non-catalytic functions were attenuated, resulting in the inhibition of ACh hydrolysis and reducing size and number of plaques. Thus, this study suggests AChE regulation is an essential role for bile acids as liver-derived molecules in the CNS. Considering the similarity between the NBML model and AD at molecular and physiological levels, CA and SDC may be potential therapeutic adjuvants for patients that are suffering from AD or other related cognitive disorders. This study additionally provides new structural

information for the rational design of new inhibitors against AChE.

ACKNOWLEDGEMENTS

This research was supported by the University of Tabriz.

REFERENCES

- Aboutwerat A, Pemberton PW, Smith A, Burrows PC, McMahon RF, Jain SK, Warnes TW (2003) Oxidant stress is a significant feature of primary biliary cirrhosis. *Biochim Biophys Acta* 1637: 142–150.
- Ackerman HD, Gerhard GS (2016) Bile acids in neurodegenerative disorders. *Front Aging Neurosci* 8: 263.
- Anand P, Singh B (2013) A review on cholinesterase inhibitors for Alzheimer's disease. *Arch Pharm Res* 36: 375–399.
- Batelli S, Albani D, Rametta R, Polito L, Prato F, Pesaresi M, Negro A, Forloni G (2008) DJ-1 modulates alpha-synuclein aggregation state in a cellular model of oxidative stress: relevance for Parkinson's disease and involvement of HSP70. *PLoS One* 3: e1884.
- Bradford MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
- Dong H, Xiang YY, Farchi N, Ju W, Wu Y, Chen L, Wang Y, Hochner B, Yang B, Soreq H, Lu WY (2004) Excessive expression of acetylcholinesterase impairs glutamatergic synaptogenesis in hippocampal neurons. *J Neurosci* 24: 8950–8960.
- Dong S, Duan Y, Hu Y, Zhao Z (2012) Advances in the pathogenesis of Alzheimer's disease: a re-evaluation of amyloid cascade hypothesis. *Transl Neurodegener* 1: 18.
- García-Ayllón MS, Small DH, Avila J, Sáez-Valero J (2011) Revisiting the role of acetylcholinesterase in Alzheimer's disease: cross-talk with P-tau and β -amyloid. *Front Mol Neurosci* 4: 22.
- Gratwicke J, Kahan J, Zrinzo L, Hariz M, Limousin P, Foltynie T, Jahanshahi M (2013) The nucleus basalis of Meynert: a new target for deep brain stimulation in dementia? *Neurosci Biobehav Rev* 37: 2676–2688.
- Holmquist M (2000) Alpha/Beta hydrolase fold enzymes: structures, functions and mechanisms. *Curr Protein Pept Sci* 1: 209–235.
- Inestrosa NC, Alvarez A, Pérez CA, Moreno RD, Vicente M, Linker C, Casanueva OI, Soto C, Garrido J (1996) Acetylcholinesterase accelerates assembly of amyloid-beta-peptides into Alzheimer's fibrils: possible role of the peripheral site of the enzyme. *Neuron* 16: 881–891.
- Johnson G, Moore SW (2006) The peripheral anionic site of acetylcholinesterase: structure, functions and potential role in rational drug design. *Curr Pharm* 12: 217–225.
- Lei J, Gao G, Feng J, Jin Y, Wang C, Mao Q, Jiang J (2015) Glial fibrillary acidic protein as a biomarker in severe traumatic brain injury patients: a prospective cohort study. *Crit Care* 19: 362.
- Liu Y, Yan B, Winkler DA, Fu J, Zhang A (2017) Competitive inhibition mechanism of acetylcholinesterase without catalytic active site interaction: study on functionalized C₆₀ nanoparticles via *in vitro* and *in silico* assays. *ACS Appl Mater Interfaces* 9: 18626–18638.
- Lo AC, Callaerts-Vegh Z, Nunes AF, Rodrigues CM, D'Hooge R (2013) Tauroursodeoxycholic acid (TUDCA) supplementation prevents cognitive impairment and amyloid deposition in APP/PS1 mice. *Neurobiol Dis* 50: 21–29.
- Mertens KL, Kalsbeek A, Soeters MR, Eggink HM (2017) Bile acid signaling pathways from the enterohepatic circulation to the central nervous system. *Front Neurosci* 11: 617.

- Moodley K, Minati L, Contarino V, Prioni S, Wood R, Cooper R, D'Incerti L, Tagliavini F, Chan D (2015) Diagnostic differentiation of mild cognitive impairment due to Alzheimer's disease using a hippocampus-dependent test of spatial memory. *Hippocampus* 25: 939–951.
- Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *J Comput Chem* 30: 2785–2791.
- Pan X, Elliott CT, McGuinness B, Passmore P, Kehoe PG, Hölscher C, McClean PL, Graham SF, Green BD (2017) Metabolomic profiling of bile acids in clinical and experimental samples of Alzheimer's disease. *Metabolites* 7: E28.
- Paxinos G, Watson C (2005) *The rat brain in stereotaxic coordinates*. 15th ed. Academic Press, San Diego.
- Prasher VP (2004) Review of donepezil, rivastigmine, galantamine and memantine for the treatment of dementia in Alzheimer's disease in adults with Down syndrome: implications for the intellectual disability population. *Int J Geriatr Psychiatry* 19: 509–515.
- Rizvi SMD, Shakil S, Haneef M (2013) A simple click by click protocol to perform docking: AutoDock 4.2 made easy for non-bioinformaticians. *EXCLI J* 12: 831–857.
- Sadeghi L, Rizvanov AA, Salafutdinov II, Dabirmanesh B, Sayyah M, Fathollahi Y (2017) Hippocampal asymmetry: differences in the left and right hippocampus proteome in the rat model of temporal lobe epilepsy. *J Proteomics* 154: 22–29.
- Sadeghi L, Yousefi Babadi V, Tanwir F (2018) Improving effects of Echium amoenum aqueous extract on rat model of Alzheimer's disease. *J Integr Neurosci* 17: 661–669.
- Seibenhener ML, Wooten MW (2012) Isolation and culture of hippocampal neurons from prenatal mice. *J Vis Exp* 65: 1–6.
- Toledano A, Alvarez MI (2004) Lesions and dysfunctions of the nucleus basalis as Alzheimer's disease models: general and critical overview and analysis of the long-term changes in several excitotoxic models. *Curr Alzheimer Res* 1: 189–214.
- Viana RJ, Ramalho RM, Nunes AF, Steer CJ, Rodrigues CM (2010) Modulation of amyloid-beta peptide-induced toxicity through inhibition of JNK nuclear localization and caspase-2 activation. *J Alzheimers Dis* 22: 557–568.
- Wenk GL (1990) Animal models of Alzheimer's disease: are they valid and useful? *Acta Neurobiol Exp* 50: 219–223.

SUPPLEMENTAL MATERIAL

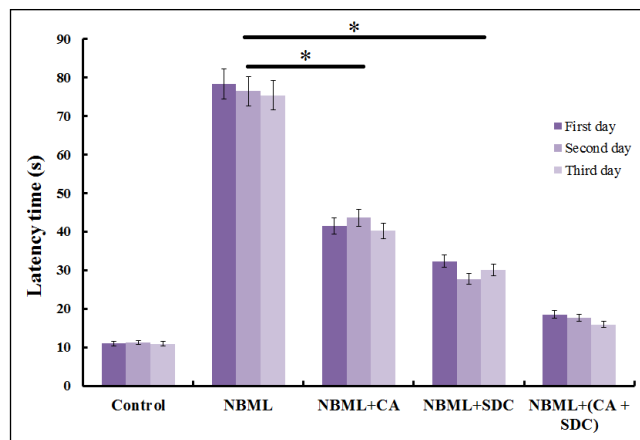


Fig. S1. Spatial learning evaluation by the Morris water maze test. The rats' latency to reach the platform increased in NBML rats in comparison with control on 3 consecutive test days. 30 mg/kg of bile acids decreased latency time for AD rats to reach the platform. Treatment with CA+SDC improved spatial navigation in NBML rats more significantly in comparison with other groups. Each data point indicates the mean \pm S.E.M. The asterisks (*) indicate significant differences ($p < 0.05$) according to Duncan's multiple range test.