Neurotoxicology 70 (2019) 122–134

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Ameliorative effect of alendronate against intracerebroventricular streptozotocin induced alteration in neurobehavioral, neuroinflammation and biochemical parameters with emphasis on Aβ and BACE-1

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ARTICLE INFO

Keywords:
Alzheimer's disease
Streptozotocin
Alendronate
Amyloid β
Neuroinflammation
BACE-1

ABSTRACT

Alzheimer's disease (AD) is the most prevalent age related neurodegenerative disorder manifested by progressive cognitive decline and neuronal loss in the brain, yet precise etiopathology of majority of sporadic or late-onset AD cases is unknown. AD is associated with various pathological events such as Aβ deposition due to BACE-1 induced cleavage of APP, neuroinflammation, increased cholesterol synthesis, cholinergic deficit and oxidative stress. It was found that bone drug, alendronate (ALN) that cross blood brain barrier inhibits brain cholesterol synthesis and AChE enzyme activity. As cholesterol modifying agents have been supposed to alter AD like pathologies, the current study was designed to investigate the possible neuroprotective and therapeutic potential of ALN against ICV STZ induced experimental sporadic AD (SAD) in mice in a non-cholesterol dependent manner, using donepezil (5 mg/kg) as a reference standard. The preliminary study was done by molecular modelling to identify the binding affinity of ALN with BACE-1 *in silico*. The prevention of cognitive impairment in mice induced by ICV STZ (3 mg/kg) infused on first and third day, by ALN (1.76 mg/kg p.o.) administered for 15 consecutive days was assessed through Spontaneous Alternation Behavior (SAB) and Morris water maze (MWM) test. Additionally, the protective effect of ALN was also observed by the reversal of altered levels of $A\beta_{1-42}$, BACE-,1 neuroinflammatory cytokines, AChE activity and oxidative stress markers (except TBARS) in ICV-STZ infused mice. However, the findings of the present study imply the therapeutic potential of ALN against SAD-like complications.

1. Introduction

Alzheimer's disease is a most complicated age-dependent progressive neurodegenerative disorder accompanied by cognitive derangement and fatal outcomes (Husain et al., 2018; Kaundal et al., 2018; Kaundal, 2018). It is considered as a major culprit for development of dementia, which is an emerging global health problem worldwide. AD is generally classified as early-onset familial AD (FAD) and late-onset sporadic AD (SAD) which is affecting majority of people (Dorszewska et al., 2016). It is manifested by iconic neuropathological hallmarks including deposition of amyloid beta (extracellular senile plaque), intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein, synaptic loss and neuronal death (Serrano-Pozo et al., 2011; Wirths et al., 2017). Neuronal loss is prominently occurred in CA1 and CA3 region of hippocampus following cerebral cortex with progression of disease (Padurariu et al., 2012). Plenty of research reported the involvement of wide range of pathological factors and several hypothesis including amyloid hypothesis in order to explain the beginning and progression of neurodegeneration in this disease (Hardy, 2006; Lee et al., 2006). Substantial number of evidences from pre-clinical and clinical studies support the key role of cholinergic dysfunction which is responsible for cognitive decline (Costa et al., 2016; Kaundal et al., 2018), high cholesterol level (Costa et al., 2018; Popp et al., 2013), oxidative stress, altered metabolic factors (Husain et al., 2018) microglia and neuroinflammation (Regen et al., 2017; Shapira et al., 2018) in generation of this severe neurodegenerative disease which is also supported by post-mortem studies of AD brain (Hayes et al., 2002). Since past decades a line of research has continuously been centered to highlight the underlying pathological mechanism but the etiopathology of AD is still ambiguous. The understanding of specific characteristics of AD requires a more in depth knowledge of brain biology as well as pathophysiological mechanisms.

Currently available therapies for AD are focusing on acetylcholinesterase (AChE) enzyme inhibition. But the AChE inhibitors

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https://doi.org/10.1016/j.neuro.2018.11.012
Received 24 September 2018; Received in revised form 22 November 2018; Accepted 22 November 2018
Available online 24 November 2018
0161-813X/

such as donepezil, rivastigmin which are supposed as golden standard confers only symptomatic relief and rises the financial burden as well (Zameer et al., 2018). Furthermore, accumulating evidences have supported the association of AD with neuroinflammatory responses (McKenzie et al., 2017; Shao et al., 2014). The release of Aβ profibrils in neurons activates microglia and evokes pro-inflammatory responses leading to the production of neurotoxic cytokines which are considerable pathogenic event in neuronal damage and progression of AD (Song et al., 2014). This imposes a need to find an alternative multi-target approach that might exhibit beneficial role by overcoming the drawbacks and checking the disease progression. Ample of new therapeutic agents such as cholesterol modifying agents, metal chelators, Aβ vaccination etc. have been examined to identify their efficacy against AD (Cibickova et al., 2007). Cholesterol modifying agents have been considered in past several years as safe and readily available therapy for AD treatment as increased cholesterol synthesis is prominently involved in pathogenesis of AD (Cibickova et al., 2009). To the same context, epidemiological data exposed the potential benefits of statins against dementia (Chu et al., 2018). Moreover, bisphosphonates (BPs) which are the recommended therapy by FDA for the treatment of bone disorders such as osteoporosis (Zameer et al., 2018), were demonstrated to be effective against dementia in osteoporosis patients (Chang et al., 2014). Alendronate, a nitrogen-containing bisphosphonate (NBP), acts by inhibiting farnesyl pyrophosphate synthase (FPPS) enzyme in Mevalonate pathway resulting in depletion of downstream components (Dunford et al., 2001). It was found to exhibit a potential to dampen the rise in AChE enzyme activity (Cibickova et al., 2007) and cholesterol synthesis in human blood (Guney et al., 2008) and rat brain (Cibickova et al., 2009). Therefore, the present study was planned to explore the molecular mechanism underlying the inhibitory potential of this NBP by performing molecular docking (in silico modelling), assessing neurobehavioral, neuroinflammatory, and oxidative stress markers with emphasis on Aβ and BACE-1 enzyme.

STZ, a glucosamine-nitrosourea compound, has been cited to induce neurodegeneration and many pathological aspects of SAD in subdiabetogenic dose comprising impaired cholinergic neurotransmission, senile plaques and intracellular neurofibrillary tangle formation via hyperphosphorylation of tau protein and cognitive dysfunctions as well (Mishra et al., 2018). In addition to these, this beta cytotoxic drug also triggers neuroinflammation and oxidative stress by altering cellular energy metabolism that further resembles the conditions reported in SAD patients (Javed et al., 2015). In the present study, by considering all these evidences, the pathologies resembling SAD was recapitulated in mice by administration of subdiabetogenic ICV dose of STZ.

1.1. Material and methods

1.1.1. Animals

Swiss albino mice of either sex weighing 25–30 g m and 6–8 weeks age were selected to perform the experiments. Mice were obtained from the Central animal house facility (Jamia Hamdard, New Delhi, India) after approval from the Institutional Animal Ethics Committee (IAEC) and were housed in a polypropylene cage in a pair of 6 mice per cage under controlled conditions of temperature and humidity (25 ± 2 °C, 55–65%) as well as presented with 12 h light and 12 h dark cycle with free access to food and water ad libitum. All the experiments following acclimatization of animals for 2–3 days at laboratory conditions were conducted in compliance with the guidelines of the Committee for the Purpose and Supervision on Experimentation on Animals (CPCSEA) under protocol number 1384.

1.1.2. Drugs and chemicals

All the drug solutions were freshly prepared prior to the experiment. Alendronate was procured from Cayman Chemicals Company, USA. Donepezil was obtained from Cipla Pharmaceutical Company, India and STZ, acetylthiocholine iodide and butyrylthiocholine iodide were purchased from Sigma Aldrich, USA. Dose of alendronate was calculated from the corresponding human dose (10 mg) recommended by FDA for the treatment of bone disorders. Mouse ELISA kits for estimation of cytokines (IL-1β, IL-6 and TNF-α) were procured from Krishgen Biosystems and BACE-1 and $A\beta_{1-42}$ were purchased from Genxbio Health Sciences Pvt Ltd and Elabscience, India respectively.

1.1.3. Treatment schedule

Mice were randomized into 7 groups comprised of 8 mice each and groups were defined as Group I (Control): Mice administered with water orally for 15 days; Group II (Sham group): Mice received citrate buffer (0.1 M, pH 4.4) ICV (5 µl bilaterally) at two alternative days; Group III (STZ infused): Mice were infused with STZ dissolved in citrate buffer at a dose of 3 mg /kg i.c.v. (5 µl bilaterally) at first and third day to induce sporadic AD; Group IV and V (STZ + Alendronate and STZ + Donepezil): Mice received ICV injection of STZ bilaterally in brain in two doses at 48 h time gap and then administered with respective dose of alendronate and donepezil for 15 days started after 24 h of second dose of STZ.; Group VI and VII (Alendronate and donepezil *per se*): Mice with oral administration of alendronate and donepezil for 15 days, respectively. Alendronate and donepezil were dissolved in water according to their respective doses prior to oral administration to mice using oral gavage. Mice treated with alendronate were not given food for 30 min following drug administration in order to avoid interference in drug absorption.

During treatment schedule of 15 days, all mice of their respective groups were subjected to behavioral paradigms started from day 9. Initially, all mice were assessed for spontaneous alternation behavior (SAB) on day 9 and then they were exposed to a training session in Morris Water Maze (day 10–14) and probe trail for long term memory assessment (day 15). After that, all mice were euthanized for collection of serum and brain tissue (hippocampus and frontal cortex). Two brain tissues from each group were stored in 4% formalin for histopathological analysis and other brain tissues as well as serum were stored at −80 °C for AChE enzyme activity (by Ellman assay), cytokines Aβ, BACE-1 and other biochemical estimations in accordance with the protocol provided with the corresponding assay kits (Fig. 1).

1.1.4. Intracerebroventricular infusion of streptozotocin

Swiss albino mice were anaesthetized with ketamine-xylazine cocktail (100 mg/kg and 20 mg/kg respectively) i.p. and the animals were placed on stereotaxic apparatus. Skull of mouse was exposed to locate the stereotaxic coordinates accurately which are -0.8 mm, ± 1.0 mm and -3.0 mm respectively, at anterio-posterior, lateral and dorso-ventral with respect to bregma and ventral from dura through a tooth bar set at 0 mm. A hole in the skull (bilaterally) was made with the help of syringe carefully in such a way that only it goes to 3 mm depth in the skull. Later on, STZ (3 mg/kg) dissolved in citrate buffer (pH 4.4) was infused into each lateral ventricle (2.5 µL each) via 28-gauge Hemilton syringe (10 µl). The sham group underwent the same stereotaxic surgery but infused with same volume of citrate buffer without STZ.

1.2. Molecular docking

The molecular docking study for assessing the binding affinity of ligand (ALN) with target protein β-site APP cleaving (BACE-1) enzyme was carried out by using 3D structure of BACE-1 enzyme retrieved from protein data bank (SC7; protein database (PDB) code: 2QP8). BACE-1 complexed with inhibito (SC7) was selected except OMM99-2 as the former inhibitor has good resolution (1.5 Å). Four BACE-1 inhibitors (LY-2886721, AZD3293, MK-8931, TAK-070) (Fig. 1), which are now engaged in clinical trials for AD and one test drug (ALN), were searched from PubChem. In the present study, automated docking with retrieved hits was performed by using Schrodinger v10 docking software suite. Molecular docking of ALN was done by removing the inhibitor SC7 and

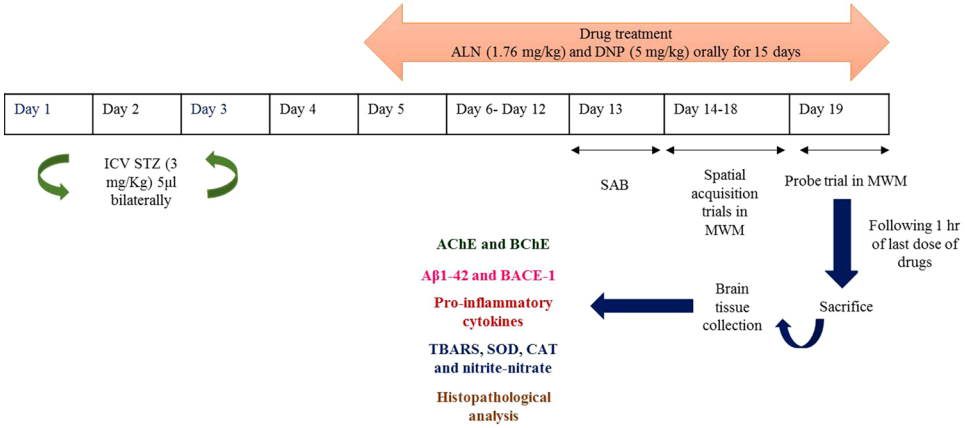

Fig. 1. Treatment schedule.
The figure depicts the experimental schedule, including drug treatment and behavioral and biochemical estimations at different time points. ICV: Intracerebroventricular; STZ: Streptozotocin; ALN: Alendronate; DNP: Donepezil; SA: Spontaneous alteration behavior; MWM: Morris water maze; AChE: Acetylcholinesterase; BChE: Butylcholinesterase; BACE-1: β-site amyloid precursor protein cleaving enzyme-1; TBARS: Thiobarbituric acid reactive substance: SOD: Superoxide dismutase and CAT: Catalase.

docking the retrieved inhibitors in the active site of BACE-1 on the basis of ligand SC7 co-ordinates. Water molecules were removed within 5 Å region as they are not essential for BACE-1 ligand interaction. Following protein preparation, ligand preparation was performed in LigPrep application of the software that resulted in fourteen structural analogues of ligands constituting the in-house library of structural analogues of BACE-1 inhibitors. Then virtual screening utilizing glide docking protocol preceding grid generation around protein was done that retain retrieved hits with better binding affinity in a good binding orientation. Glide docking ranked the molecules which are favorably docked as BACE-1 inhibitors according to their docking score. The compounds were docked three times and then the results were evaluated on the basis of docking score and hydrogen bond formation with surrounding amino acids that predict their binding and proper alignment towards active site of target protein (Noolvi et al., 2012)

1.3. Behavioral paradigms

1.3.1. Spontaneous alternation behavior

Spontaneous alternation behavior was assessed by employing wooden cross maze consisting of four open arms (length 23.5 cm, breadth 8 cm, wall height 10 cm) and a central platform (8 × 8 cm). This four-arm wooden maze was made similar in dimensions to elevated plus maze with the difference that this cross maze did not have two closed arms. The maze was elevated at a height of 50 cm to avoid escape of animals. During the experiment mice were gently place on the central platform and allowed to traverse all four arms of the maze freely for 6 min and the number and sequence of arm entries were noted throughout the experiment. An alternation was defined as entry into four different arms on overlapping quintuple sets. The percentage alternation was calculated as the ratio of actual alternation/possible alternation × 100. Actual alternation represented the total number of quintuple sets which comprised of a set of five consecutive arm choices within the total set of arm choices (ABCDA was a quintuple while BCDDC was not as the entry in arm A is not included). The possible alternation is equal to the total number of arm entries minus 4. (Vohora et al., 2005; Zameer and Vohora, 2017)

1.3.2. Morris water maze test

The Morris water maze was conducted to assess the learning capacity of animals to navigate to a particular location in a large spatial environment. This task was performed in Panlab Harvard apparatus with SMART v3.0.03 software from USA. Morris water maze comprises of large stainless steel circular tank (122 cm in diameter and 51 cm height) filled with water and equipped with non-reflecting interior surface for mice. A circular plexiglass platform (10–12 cm in diameter), which was made hidden by generating a completely invisible background-to-platform color match, was submerged at a level of 0.5–1 cm below the surface of water filled in a tank. Water was maintained at ambient temperature of 19–22 °C in order to avoid hypothermia to mice and other conditions such as light and sound was also maintained to avoid any error. To measure spatial learning and memory, surrounding cues (different shapes of paper on tank wall) were used to assist mice to follow for escaping to the target.

For spatial acquisition task, water pool was divided virtually into four equal quadrants by designing two principle axis and were named as north, east, south and west. The escape platform was located in one quadrant (south-west) and remains fixed in the same quadrant throughout the experiment. In this task, each mouse underwent four sequential trials with inter-trial interval of 30 s in each session for 5 days. Each mouse was positioned in one quadrant (different quadrant for each trial) opposite to the pool wall and allowed to navigate the spatial environment in order to find the hidden platform (target) within a celling time of 60 s. Mouse, prior to exposure to co-trial of the session, was allowed to sit onto the platform for 30 s. In case of failure of mouse to escape to the target within the maximum escape time of 60 s, it was gently directed onto the hidden platform and allowed to remain there for the same trail duration. During each trial, the escape latency which is defined as the time required by mouse to allocate to the hidden platform as well as path length that is the distance traversed by each mouse to reach the platform was noted for 5 days.

On sixth day, a probe trial was performed for assessment of reference memory. In this trial platform was removed from the pool and mouse was exposed to the water maze by placing in the novel quadrant (opposite to the initial platform location). Each mouse was allowed to swim freely for 60 s. The dwell time which is the time spent in the target quadrant (where platform was earlier located) was recorded and it represented the memory consolidation following learning. (Vorhees and Williams, 2006; Zameer and Vohora, 2017)

1.4. Biochemical parameters

1.4.1. Brain tissue homogenate preparation

Hippocampus and frontal cortex, obtained following euthanasia of mice, were rinsed with ice-cold saline and then homogenized in chilled phosphate buffer (0.1 M; pH 7.4) (10 times the volume of tissue). Homogentaes were centrifuged at 800 × *g* at 4 °C for 5 min. The obtained supernatant was again centrifuged at 10,000*g* at 4 °C for 10 min to collect the post-mitochondrial (PMS) supernatant for estimation of cholinesterases (AChE and BChE) and oxidative stress biomarkers.

1.4.2. Estimation of cholinesterases (ChEs) enzyme activities

The increase in ChEs (AChE and BChE) enzyme activities has been cited to attenuate level of acetylcholine (ACh) by promoting its hydrolysis (Remya et al., 2014). The enzyme activities were assessed by Ellman's assay with some modification on the basis of rate of formation

of thiocholine from acetylthiocholine and butyrylthiocholine iodide in the presence of AChE and BChE respectively, which was measured by using spectrophotometer. For AChE estimation, reaction mixture containing 0.2 ml acetylthiocholine iodide (75 mM), 0.1 ml buffered Ellman's reagent DTNB (10 mM in 15 mM NaHCO3) and 3 ml PBS (25 mM, pH 7.4) was incubated at room temperature for 10 min. Similarly, BChE activity was analyzed by adding 0.2 ml butyrylthiocholine iodide in 0.1 ml Ellman's reagent (10 mM DTNB) following addition of PBS upto volume 3 ml. Following incubation 0.2 ml enzyme sample or brain homogenate was added. The optical density was observed at 412 nm spectrophotometrically within 5 min. AChE and BChE enzyme activities were measured as optical density/mg protein. (Ellman et al., 1961; Reeta et al., 2017; Wang, D. et al., 2018)

1.4.3. Estimation of $A\beta_{1-42}$ and BACE-1 enzyme

The quantification of $A\beta_{1-42}$ and BACE-1 enzyme was done by using a mouse ELISA kit according to the instructions provided by Elabsciences and Genxbio Health Sciences Pvt Ltd respectively. The quantification employs the sandwich enzyme immunoassay technique. A monoclonal antibody specific for mice $A\beta_{1-42}$ or BACE-1 has been precoated in the microplate. Standards, control and samples were pipetted into the wells following incubation for the duration given in the instructions of specific kit, and any mice $A\beta_{1-42}$ or BACE-1 present is bound by the immobilized antibody. After that washing was performed using a wash buffer to remove any unbound substance in the wells and then an enzyme-linked polyclonal antibody specific for mice $A\beta_{1-42}$ or BACE-1 was added to the wells. Following incubation unbound antibody enzyme reagent was removed by washing buffer and a substrate solution was added into the wells. The enzyme reaction developed a blue color that turns yellow in addition of stop solution. The intensity of color measured spectrophotometrically is proportional to the amount of mice $A\beta_{1-42}$ or BACE-1 bound in the initial step. The sample values were then interpreted from the standard curve. Values were expressed as mean ± S.E.M after statistical analysis.

1.4.4. Estimation of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α)

The pro-inflammatory cytokines (IL-6, IL-1β and TNF-α in hippocampus and frontal cortex of mice of all experimental groups was estimated by using mice IL-6, IL-1β and TNF-α ELISA kit procured by Krishgen Biosystems. The assay was based on principle of the sandwich ELISA technique and performed according to the instructions given with the specific cytokine kit. The samples were initially allowed to bind with the pre-coated antibody in the wells. After incubation, the unbound substances were removed by washing and then an enzyme-linked polyclonal antibody specific for mice IL-6, IL-1β and TNF-α was added. After washing to remove the unbound antibody enzyme reagent, substrate solution was added that develop a blue color. The reaction was stopped by adding stop solution that turns blue color to yellow. The absorbance was taken by using a spectrophotometer. The color intensity indicates the amount of IL-6, IL-1β and TNF-α present in the sample.

1.5. Oxidative stress biomarkers

1.5.1. Estimation of lipid peroxidation

The level of malondialdehyde (MDA) has been reported to be assessed as an index of lipid peroxidation. The MDA level was determined by using the method of Wills. Accordingly, 0.5 ml PMS mixed with 0.5 ml of tris-HCL was incubated for 2 h at 37 °C. After incubation, 1 ml trichloroacetic acid (TCA, 10%) was added and centrifuged at 1000 × *g* for 10 min. 1 ml of supernatant thus obtained was mixed with 1 ml of thiobarbituric acid (TBA 0.67%). The tubes containing reaction mixture were placed in boiling water for 10 min. After cooling 1 ml distilled water was added and then absorbance was measured at 532 nm. TBARS (thiobarbituric acid reactive substances) were quantified using extinction co-efficient of $1.56 \times 10^5 M^{-1}$. TBARS were expressed as nmol MDA/mg protein. (Sachdeva and Chopra, 2015; Wills, 1966)

1.5.2. Catalase (CAT)

CAT activity was determined following the procedure of Calibrne (1985). Briefly, 1.95 ml phosphate buffer (0.5 M, pH 7.0) was added to the reaction mixture consisting of 0.05 ml PMS and 1 ml of hydrogen peroxide (H_2O_2 0.019 M) to make the final volume upto 3 ml. Change in absorbance was noted at 240 nm to assess the CAT activity which was calculated in terms of nmol H2O2/min/ mg protein. (Ayyub et al., 2017; Sachdeva and Chopra, 2015)

1.5.3. Superoxide dismutase (SOD)

The total activity of SOD enzyme was determined following method of Markland and Markland by incorporating some modifications (Marklund and Marklund, 1974). The determination is based on the ability of SOD to inhibit the autoxdation of pyrogallol. 10 µl tissue homogenate was mixed with 970 µl of buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.2) following addition of 20 µl of pyrogallol (13 mM) at room temperature. Following the initial lag time of 30 s, the samples were analyzed in triplicates by spectrophotometric method at 420 nm to demonstrate the changes in absorbance. One unit SOD activity is defined as the amount of enzyme that inhibited autoxidation of the total pyrogallol by 50% per minute and it was expressed as U/min/mg protein. (Marklund and Marklund, 1974; Tung et al., 2017)

1.5.4. Estimation of nitrite-nitrate level

The estimation of NO level is based on the formation of its stable products in the form of nitraite and nitrate. NO level was determined in freshly prepared brain tissue homogenate by using Griess reagent. To perform the assay equal parts of tissue supernatant was added with Griess reagent (0.1% N-(-naphthyl ethylenediamine dihydrochloride, 1% sulfanilamide and 5% phosphoric acid). (Richa et al., 2017; Wu and Yotnda, 2011).

1.6. Protein estimation

Total protein concentration in each hippocampus and frontal cortex was estimated by Lowry's method by using bovine serum albumin as a standard. (Lowry et al., 1951)

1.7. Cholesterol estimation

Cholesterol level was analyzed in mice brain tissue by using method of Cibickova and colleagues with some modifications (Cibickova et al., 2009). Hippocampus of mice brain were homogenized and extracted following the protocol of Bilgh and Dyer (Bligh and Dyer, 1959). Briefly, homogenized samples of different groups were mixed with methanol and water solution (methanol: water - 2:0.8) and extracted further to chloroform. The chloroform layer was separated and evaporated to dryness. Cholesterol was then derivatized using acetylchloride solution in chloroform (1:5) for one hour. The mixture was evaporated under nitrogen following dissolution of residue containing cholesterol acetate in n-hexane for analysis. The stock solution of standard cholesterol was prepared in hexane at 5 mg/ml concentration. Different dilutions were made from the stock solution ranging from 0.05 to 10 µg/ml. The standard and samples were injected and analyzed in GC–MS system operating in electron ionization mode. The temperature of injector, oven and ionization source was set 300 °C, 320 °C isothermally and 280 °C respectively and the slit ratio were set to 1:10. The ions *m/z* 367, 369 and 370.6 were recorded. The concentration of cholesterol in brain tissue was estimated by linearity slope (Cibickova et al., 2009).

1.8. Congo red staining for Aβ deposition

A well-established method of Hou and co-workers was employed for

Congo red staining of mice hippocampal tissue. The mice brains were dissected and hippocampal sections were made with the help of microtome. The brain tissue was fixed in 4% paraformaldehyde for 48 h and then dehydrated in 20% and 30% sucrose respectively. Tissue sections from the right hemisphere were prepared with the microtome. The sections were dipped in Congo red solution for 18 min following washing in running tap water for 20 min. The sections were then placed in weak alkali for 10 s and incubated in hematoxylin for 5 min. The tissue sections were washed again in running tap water for 30 min and de-stained in weak acid for several seconds. Prior to dipping in eosin for 15 min sections were washed for 20 min. Eosin dipped sections were again washed for 15 min in running water and then dehydrated in an alcohol gradient. At the end xylene clearance and cover slipping was done. Aβ deposition was observed under a Moticam light microscope. (Hou et al., 2008; Singh et al., 2013)

1.9. Statistical analysis

The statistical analysis of the data obtained from behavioral and biochemical parameters following STZ and drug treatment in mice was performed by using GraphPad Prism software version 5.01. The data were represented as mean ± SEM. Student-Newman Kewl's test in SAB, repeated measure ANOVA in escape latency; path length and speed in MWM and one-way ANOVA in all other parameters followed by post-hoc Tukey-Multiple Comparison test was used.

2. Results

2.1. Molecular docking analysis

In molecular docking studies the results were interpreted by docking and score interacting amino acid residues at active site of target protein with ligand via hydrogen bonds. The lower docking score assess the strength of ligand interaction with target. The test drug ALN was observed to have interactions with ASP289, ASP93, GLY95, GLY91, THR133, TYR259 residues residing in BACE-1 binding domain. The docking score of ALN was found to be -6.491 which was lower than other selected BACE-1 inhibitors showing favorable binding mode to BACE-1 enzyme (Fig. 2).

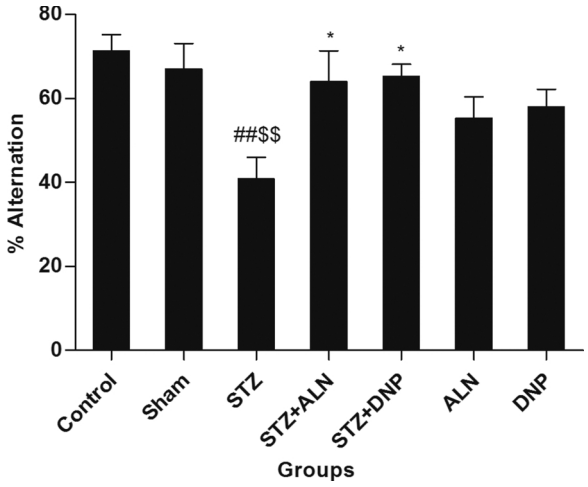

Fig. 3. Spontaneous alternation behavior in ICV STZ induced AD in mice.
ALN was found to reverse the behavioral deficit induced by STZ in spontaneous alternation behavior test. The % alternation was significantly increased in drug treated animals as compared to STZ treated animals (toxic group) as analyzed by one-way ANOVA followed by post-hoc Student-Newman keul's test. The data are represented as Mean ± SEM. $^{\$\$}p < 0.01 indicates comparison with control, $^{\#\#}p < 0.01$ with sham and $^{*}p < 0.05$ with STZ (toxic) group. STZ: STZ: Streptozotocin; ALN: Alendronate and DNP: Donepezil.

2.2. Spontaneous alternation behavior test

ICV STZ (3 mg/kg) infused on first and third day showed significant increase in % alternation as compared to control and sham (df = 6, 49 F = 3.893 p < 0.01) (p < 0.01) (statistically measured by one-way ANOVA). Post-hoc Student-Newman Keul's test indicated significant reduction in % alternation in mice of groups IV and V. This gives a clue that ALN could prevent ICV STZ impaired cognitive deficit (Fig. 3).

2.3. Morris water maze test

2.3.1. Spatial acquisition

2.3.1.1. Escape latency. MWM test is used to assess the spatial learning and memory in mice following 5 days training session constituting 4 trials each day. The escape latencies to reach the target platform

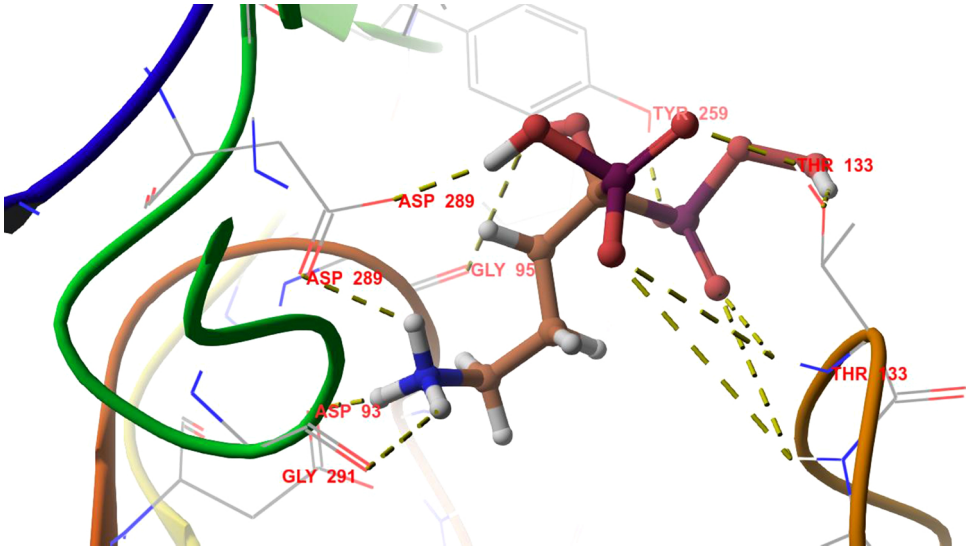

Fig. 2. Molecular docking.
The figure depicts the binding orientation of alendronate (ALN) in the active sites of BACE-1. Dotted lines indicate hydrogen bond interactions of ligand with residues of BACE-1.

decreased gradually during 5 days training session except on day 1 ($df = 6, 42\,F = 0.9950\ p > 0.05$), in spatial acquisition in MWM in all groups revealing learning except those belonging to ICV STZ. Mice infused with STZ acquired more time escape to the submerged platform as compared to control and sham groups ($df = 6, 42\,F\ 7.158\ p < 0.001$). Oral administration of drugs (ALN and DNP) for 15 days in ICV STZ mice attenuated significantly the escape latency and path length as compared to STZ group ($p < 0.01$) (Fig. 4A).

2.3.1.2. Path length. Path length that is the total distance travelled to find the hidden target was significantly increased in STZ infused mice during training session (but not on day 1 ($df = 6, 42\,F = 0.396$ p.0.05) as compared to control and sham groups ($p < 0.001$) as analyzed by repeated measure ANOVA. Treatment with ALN and DNP in STZ infused mice observed to reach the hidden platform with significantly reduced path length on day 4 and 5 as compared to that of ICV STZ group ($p < 0.01$) (Fig. 4B).

2.3.1.3. Mean speed in zone. During 5 days training trials, no significant differences among mice of ICV STZ, control and sham groups observed as measured by repeated measure ANOVA ($p > 0.05$). Post-hoc Tukey Kramer's Multiple comparison test showed no differences among drug treated groups and STZ group indicating no effect on motor activity of mice ($df = 6, 42\,F = 0.3789\ p > 0.05$) (Fig. 4C).

2.3.1.4. Reference memory test. In probe trial performed 24 h later to spatial acquisition, mice in ICV STZ group failed to memorize the location of platform, showed significantly less % dwell time as compared to control and sham groups ($p < 0.01$) inferring less time spent in target quadrant where platform was previously located. Moreover, ALN- and DNP-treated STZ infused mice found to have a significantly increased % dwell time compared to those of ICV STZ ($p < 0.05$) as analyzed by one-way ANOVA followed by post-hoc Tukey Kremer Multiple comparison test indicating improved consolidation and long-term memory ($df = 6, 49\,F = 3.878\ p < 0.01$) (Fig. 4D).

2.4. Effect of ALN on ChEs (AChE and BChE) enzyme activities

There was a significant increase in ChEs (AChE and BChE) enzyme activities in hippocampus and frontal cortex of mice brain following ICV administration of STZ (3 mg/kg bilaterally) as compared to control and sham group ($df = 6, 35\,F = 4.510\ p < 0.01$ and $df = 6, 35\,F = 3.966\ p < 0.01$ respectively). STZ infused mice treated with ALN and DNP (1.76 and 5 mg/kg/day p.o. respectively) for 15 days were observed with significant reduction in AChE but not BChE enzyme activity in hippocampus as well as frontal cortex as compared to those treated with STZ ($p < 0.05$). The results were analyzed by one-way ANOVA followed by post-hoc Tukey-Kramer Multiple Comparison test (Fig. 5A, B).

2.5. Effect of ALN on amyloid Beta ($A\beta_{1\text{-}42}$)

To investigate the anti-amyloid genesis effect of ALN, The inhibitory

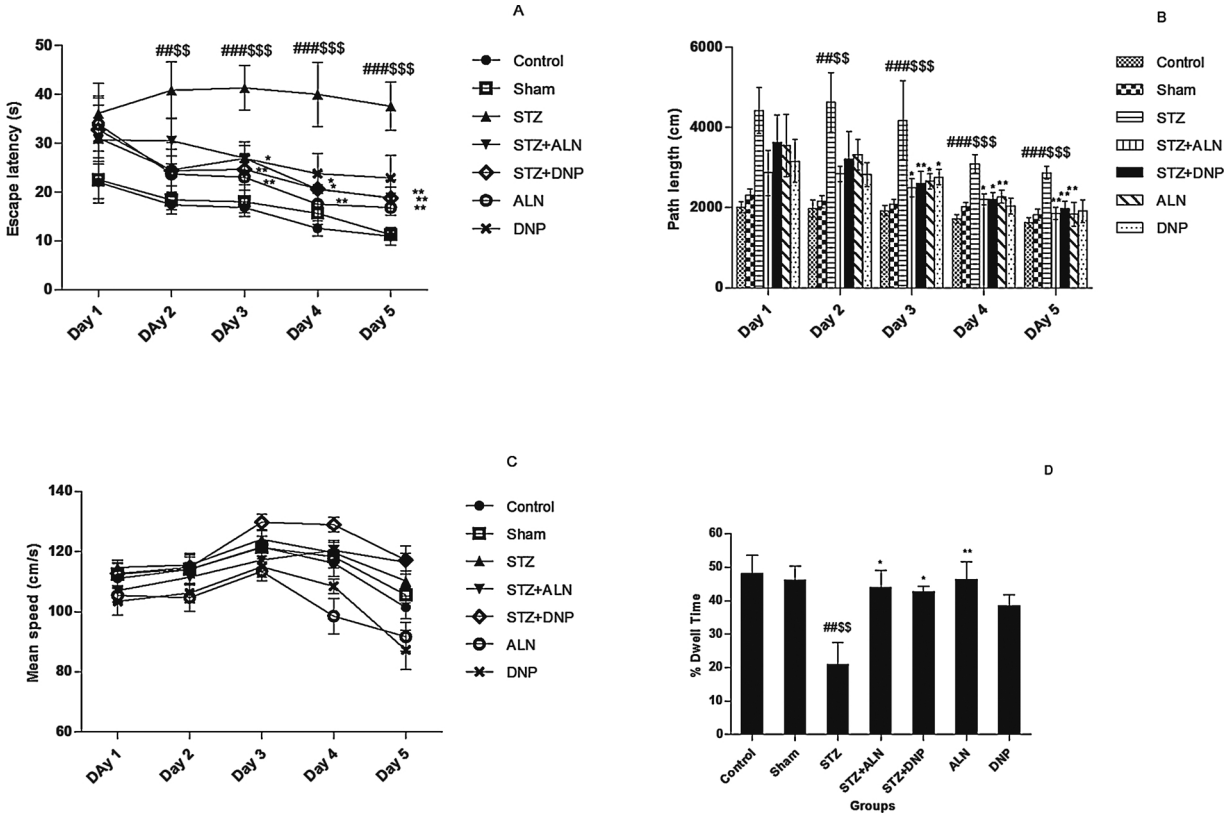

Fig. 4. Morris water maze test in ICV STZ induced AD in mice.
This figure shows A. escape latency, B. path length and C. mean speed assessed during spatial acquisition trial and D. % dwell time in probe trial in Morris water maze. There was acquisition phase for 5 days constituting 4 trials each day with 15 s inter-trial interval and data point gives the mean of 4 trials. The escape latency and path length were subsequently decreased from day 3 to day 5 and % dwell time was increased in ALN and DNP treated groups as compared to STZ treated group. On the contrary, no change was observed in mean speed in any group. The differences were found to be significant as analyzed by repeated measure ANOVA (acquisition phase) and one-way ANOVA (reference memory phase) followed by *post-hoc* Tukey-Kramer Multiple Comparison test. Data are given as mean ± SEM. The markers indicate the difference $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ when compared to control, $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ when compared to sham and $^{*}p < 0.05$; $^{**}p < 0.001$ when compared to STZ (toxic) group. STZ: Streptozotocin; ALN: Alendronate and DNP: Donepezil.

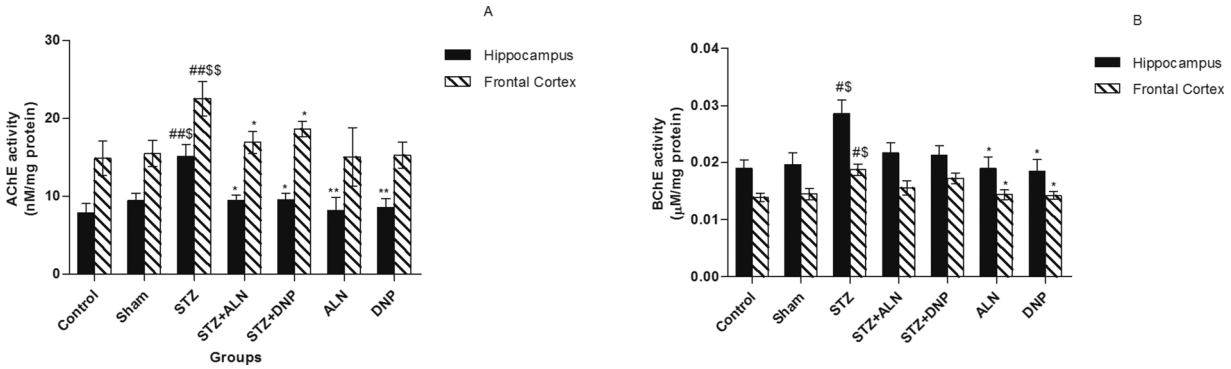

Fig. 5. Effect of ALN on ChEs enzyme activities in ICV STZ induced AD in mice.
The figure shows the effect of ALN on A. AChE and B. BChE enzyme activity in brain tissue in ICV STZ injected mice. The drug treated mice were observed with significant inhibition of AChE enzyme activity in hippocampus (but not in frontal cortex) which was augmented by ICV STZ injection. BChE activity was increased in ICV STZ mice in comparison to other groups but drug treatment did not show any significant change in this enzyme activity in both brain tissues. The results were statistically analyzed by one-way ANOVA followed by *post-hoc* Tukey-Kramer Multiple Comparison test. The values of enzyme activity are mean ± SEM $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ as compared to control; $^{\$}p < 0.05$ as compared to sham and $^{*}p < 0.05$ as compared to STZ (toxic) group. ICV: Intracerebroventricular; STZ: Streptozotocin; ALN: Alendronate and DNP: Donipezil; AChE: Acetylthiocholinesterase and BChE: Butylcholinesterase.

effect on $A\beta_{1\text{-}42}$ level in mice different brain structures was examined using mouse $A\beta_{1\text{-}42}$ ELISA kit. The STZ treated mice showed significantly increased concentration of $A\beta_{1\text{-}42}$ in hippocampus as well as frontal cortex when compared to control and sham group ($p < 0.001$). STZ injected mice following 15 days oral administration of ALN and DNP were observed with significant reduction in concentration of Aβ1-42 as compared to STZ group in both hippocampus ($df = 6$, $35\,F = 7.444$ $p < 0.01$) and frontal cortex ($df = 6$, $35\,F = 6.704$ $p < 0.05$) as indicated by one-way ANOVA. This was followed by post-hoc Tukey-Kramer Multiple Comparison test that indicated no significant changes among control, sham and drug per se groups (Fig. 6).

2.6. Effect of ALN on BACE-1 enzyme activity

The β-site amyloid precursor protein cleaving enzyme-1 (BACE-1), a key enzyme in amyloid genesis pathway, was examined by using mouse BACE-1 ELISA kit in different brain tissues after treatment of ALN, DNP and STZ to their respective groups. When compared with control and sham group, mice intracerebroventrically injected with STZ at a dose of 3 mg/kg bilaterally showed significant increase in concentration of BACE-1 in different brain tissues (hippocampus: $df = 6$, $35\,F = 4.896$ $p < 0.01$ and frontal cortex: $df = 6$, $35\,F = 4.984$ $p < 0.01$). On the other hand, oral administration of ALN significantly reversed the STZ induced-increase in concentration of BACE-1 enzyme in hippocampus and frontal cortex as well. The statistical differences were carried out by one-way ANOVA following multiple group comparison by Tukey-Kramer Multiple Comparison test (Fig. 7).

2.7. Effect of ALN on neuroinflammatory cytokines

Control, Sham-operated citrate buffer inject and ALN and DNP per se groups had not shown any significance difference in levels of proinflammatory cytokines among themselves. On the contrary, ICV dose of STZ significantly elevated level of IL-6, IL-1β and TNF-α in mice hippocampus and frontal cortex as compared to control and sham group ($p < 0.05$). However, ALN and DNP administration and their respective combination with STZ significantly attenuated IL-6, IL-1β and TNF-α level in selected brain tissues as compared to STZ treated group

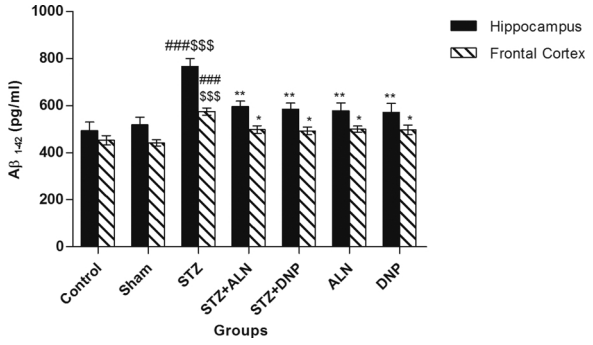

Fig. 6. Effect of ALN on $A\beta_{1\text{-}42}$ in ICV STZ induced AD in mice.
Effect of ALN (1.76 mg/kg/day p.o.); DNP (5 mg/kg/day p.o.) and STZ (3 mg/kg ICV) on $A\beta_{1\text{-}42}$ concentration measured in mice brain tissues (hippocampus and frontal cortex) by using ELISA kit. The STZ induced increase in concentration of $A\beta_{1\text{-}42}$ in mice brain tissues was significantly attenuated by oral administration of ALN and DNP. Statistical analysis was carried out by one-way ANOVA followed by *post-hoc* Tukey-Kramer Multiple Comparison test and shows the mean ± SEM values of 6 mice. The asterisks $^{\#\#\#}p < 0.001$ represent the statistical difference from control, $^{\$\$\$}p < 0.001$ from sham and $^{*}p < 0.05$; $^{**}p < 0.01$ from STZ (toxic) group. ICV: Intracerebroventricular; STZ: Streptozotocin; ALN: Alendronate; DNP: Donipezil; $A\beta_{1\text{-}42}$: Amyloid beta; and p.o. per oral.

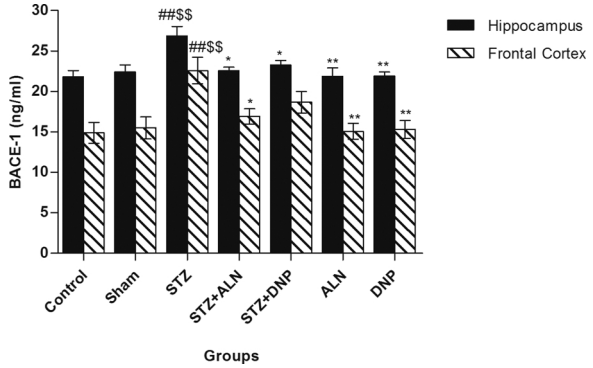

Fig. 7. Effect of ALN on BACE-1 in ICV STZ induced AD in mice.
Effect of ALN and DNP treatment on BACE-1 level in hippocampus and frontal cortex following ICV administration of STZ in mice using ELISA Kit Mice treated with oral dose of ALN (1.76 mg/kg/day) and DNP (5 mg/kg/day) for 15 days showed significant reduction in level of BACE-1 in selected brain tissues as analyzed by one-way ANOVA followed by *post-hoc* Tukey-Kramer Multiple Comparison test. Each data point is the mean of 6 mice and indicated as mean ± SEM. The statistical markers $^{\#\#}p < 0.01$; $^{\$\$}p < 0.01$ and $^{*}p < 0.05$; $^{**}p < 0.01$ show comparison with control, sham and STZ (toxic) group respectively. STZ: Streptozotocin; ALN: Alendronate; DNP: Donipezil and BACE-1: β-site amyloid precursor protein cleaving enzyme-1.

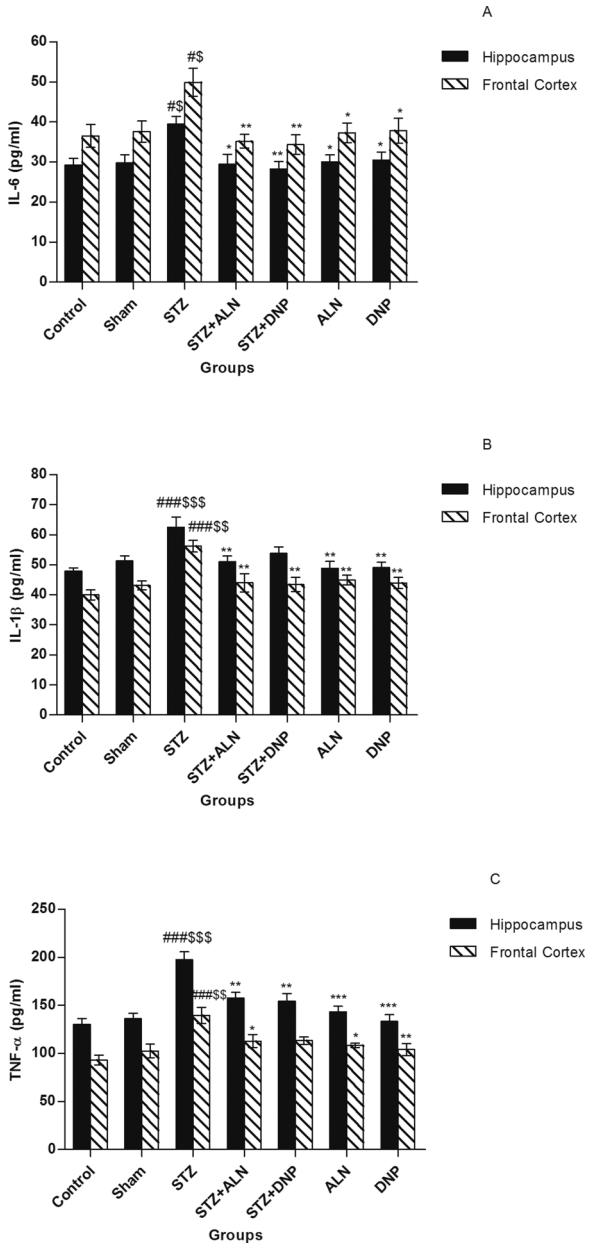

Fig. 8. Effect of ALN on proinflammatory cytokines in ICV STZ induced AD in mice.

Effect of ALN following 15 days oral administration on A. IL-6, B. Il-1β and C. TNF-α in streptozotocin injected mice. There was a significant decrease in levels of proinflammatory cytokines (IL-6, IL1β and TNF-α) in both hippocampus and frontal cortex of mice treated with ALN as compared to STZ. Values are given in mean ± SEM. The statistical analysis, one-way ANOVA followed by *post-hoc* Tukey-Kramer Multiple Comparison test, shows the comparison of all groups $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ vs control, $^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$ vs sham and $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs STZ (toxic) group. STZ: Streptozotocin; ALN: Alendronate and DNP: Donipezil.

($p < 0.05$). Mice receiving STZ + DNP were not found to have any significant alteration on levels of Il-1β in hippocampus and TNF-α in frontal cortex. The significant differences were analyzed by one-way ANOVA followed by *post-hoc* Tukey Multiple Comparison test ((IL-6: df = 6, 35 F = 3.770 $p < 0.001$; IL-1β: df 6, 35 F = 5.662 $p < 0.001$; TNF-α: df 6, 35 F = 11.51 $p < 0.001$ in hippocampus and IL-6: df = 6, 35 F = 3.702 $p < 0.01$; IL-1β: df 6, 35 F = 6.304 $p < 0.001$; TNF-α: df 6, 35 F = 5.865 $p < 0.001$ in frontal cortex) (Fig. 8A, B).

2.8. Effect of ALN on oxidative stress biomarkers (TBARS, CAT, SOD and NO)

To understand the ameliorative effect of ALN on oxidative stress in different parts of brain levels of thiobarbituric acid reactive substances (TBARS), catalase (CAT), superoxide dismutase (SOD) and NO were measured. The degree of free radical damage induced by ICV administration of STZ was assessed using lipid peroxidation which was expressed in terms of TBARS level. The TBARS and NO showed a significantly higher level in hippocampus and frontal cortex of mice belonging to STZ group as compared to control and sham group ($p < 0.05$). But administration of ALN and DNP for 15 days had no significant alteration in level of TBARS in both hippocampus (df 6, 35 F = 2.749 $p > 0.05$) and frontal cortex (df 6, 35 F = 4.00 $p > 0.05$) while ALN was found to induce significant reduction in NO level in hippocampus (df 6, 35 F = 4.778 $p < 0.05$ (but not frontal cortex df 6, 35 F = 3.675 $p > 0.05$).

On the contrary, the activity of CAT and SOD in both brain tissues was significantly attenuated in STZ treated mice as compared to control and sham group ($p < 0.05$). However, the activity of these enzymes had a notable increase in activity in hippocampus as well as frontal cortex of mice following treatment with ALN and DNP as indicated by one-way ANOVA (CAT: df 6, 35 F = 3.639 $p < 0.05$; SOD: df 6, 35 F = 3.907 $p < 0.05$ in hippocampus and CAT: df 6, 35 F = 3.888; SOD: df 6, 35 F = 4.619 $p < 0.05$ in frontal cortex). The post-hoc Tukey-Kramer Multiple Comparison test analyzed no significant difference among control, sham and drug per se (ALN/DNP per se) groups (Table 1).

2.9. Effect of ALN on cholesterol level

ICV STZ infused mice hippocampus was observed with no significant change in cholesterol level in comparison to control, sham and drug treated groups ($p > 0.05$). Regardless of cholesterol lowering drug, ALN treated STZ infused mice have not shown any change in cholesterol level (df 6, 35 F = 0.2960 $p > 0.05$) (Table 2).

2.10. Histopathological analysis

Histopathological analysis of hippocampus of mice belonging to different experimental groups was carried out by Congo red staining specific for Aβ. Hippocampal tissue was selected as prominent changes in Aβ level ELISA were quantified by ELISA. Increased Congo red staining was observed in CA1 and CA3 region in hippocampus of mice infused with twice dose of STZ (on first and third day) as compared to those of control, sham and drugs per se groups. Treatment with ALN significantly reduced Congo red staining indicating attenuation of increase in Aβ deposition in hippocampus following ICV STZ. No changes in CA1 and CA3 region of hippocampus of control, sham and drugs per se groups were observed (Fig. 9).

3. Discussion

Alzheimer's disease, an irreversible neurodegenerative disease leads to the deterioration of brain functions and is known to affect millions of people across the globe. Symptoms of this neurodegenerative disorder such as severe dementia occur over a long period of time that results in a need of developing the therapeutic molecules that should retard the disease progression by targeting this silent duration of the disease. Intracerebroventrically administered STZ in subdiabetogenic dose in mice is a well-known non-transgenic mice model of sporadic AD due to generation of pathologies similar to SAD including cognitive deficit, Aβ deposition, neuroinflammation, oxidative stress etc (Fig. 10).

In the present study, we performed molecular docking studies in order to predict the binding affinity of selected drug ALN with BACE-1 enzyme. Following this in silico modelling, in vivo experiment was

Table 1
Effect of ALN on oxidative stress biomarkers in ICV STZ induced AD in mice.

Groups	N	Treatment	Dose	Oxidative stress biomarkers in Hippocampus			
				TBARS (nmol MDA/mg protein)	CAT (nmol H_2O_2/min/mg protein)	SOD (U/min/mg protein)	Nitrite-nitrate (μmol/ml)
A. In hippocampus							
1	8	Water (Control)	10 ml/kg p.o.	1.209 ± 0.47	13.93 ± 0.69	8.726 ± 0.60	18.47 ± 1.01
2	8	Citrate Buffer (Sham control)	5 μl i.c.v.	1.636 ± 0.32	12.36 ± 0.53	8.214 ± 0.46	19.52 ± 1.21
3	8	Streptozotocin	3 mg/kg i.c.v.	2.555 ± 0.48$^{\#\$}$	7.117 ± 1.01$^{\#\#\$}$	5.292 ± 0.72$^{\#\#\$}$	25.32 ± 1.43$^{\#\#\$}$
4	8	Streptozotocin + Alendronate	3 mg/kg i.c.v. + 1.76 mg/kg p.o.	1.310 ± 0.45	12.55 ± 0.60*	8.332 ± 0.90*	19.91 ± 0.78*
5	8	Streptozotocin + Donepezil	3 mg/kg i.c.v. + 5 mg/kg p.o.	1.504 ± 0.33	12.78 ± 1.18*	8.546 ± 0.50*	19.77 ± 1.03*
6	8	Alendronate per se	1.76 mg/kg p.o.	1.945 ± 0.61	12.39 ± 1.23*	8.745 ± 0.42**	18.78 ± 1.08**
7	8	Donepezil per se	5 mg/kg p.o.	1.517 ± 0.45	11.24 ± 2.05	8.697 ± 0.64**	18.13 ± 1.17**
B. In frontal cortex							
1	8	Water (Control)	10 ml/kg p.o.	1.620 ± 0.34	12.87 ± 1.48	8.744 ± 0.59	17.69 ± 1.03
2	8	Citrate Buffer (Sham control)	5 μl i.c.v.	1.836 ± 0.39	12.57 ± 0.94	7.965 ± 0.51	18.32 ± 0.64
3	8	Streptozotocin	3 mg/kg i.c.v.	2.877 ± 0.28$^{\#\$}$	6.501 ± 0.79$^{\#\#\$\$}$	5.333 ± 0.49$^{\#\#\$}$	23.90 ± 2.34$^{\#\$}$
4	8	Streptozotocin + Alendronate	3 mg/kg i.c.v. + 1.76 mg/kg p.o.	2.929 ± 0.46	11.77 ± 1.00*	7.975 ± 0.71*	21.32 ± 1.01
5	8	Streptozotocin + Donepezil	3 mg/kg i.c.v. + 5 mg/kg p.o.	1.803 ± 0.24	11.55 ± 1.32*	8.233 ± 0.41**	19.46 ± 0.81
6	8	Alendronate per se	1.76 mg/kg p.o.	1.909 ± 0.31	12.37 ± 1.10*	8.421 ± 0.35**	17.71 ± 0.67*
7	8	Donepezil per se	5 mg/kg p.o.	1.680 ± 0.25	11.61 ± 0.92*	7.661 ± 0.50*	18.21 ± 1.05*

This represents the effect of STZ, ALN, DNP on TBARS, CAT, SOD and nitrite-nitrate level in mice A. hippocampal and B. frontal cortex tissue. The drug administration significantly reversed the oxidative stress induced by ICV STZ in mice by elevating levels of CAT, SOD, GSH (except TBARS) and decreasing nitrite-nitrate level in hippocampus (not in frontal cortex). Data are given as mean ± SEM. Markers represent the difference $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ when compared to control; $^{\$}p < 0.05$; $^{\$\$}$p,0.01 when compared to sham and $^{*}p < 0.05$ when compared to STZ (toxic) group as analyzed by one-way ANOVA followed by post-hoc Tukey-Kramer Multiple Comparison test. TBARS: Thiobarbituric acid reactive substance: CAT: Catalase and SOD.

conducted in which subdiabetogenic ICV dose of STZ was found to deplete learning and memory in mice as well as altered iconic hallmarks of SAD including $A\beta_{1\text{-}42}$, pro-inflammatory cytokines ChEs and BACE-1 enzyme as well as oxidative stress markers in hippocampus and frontal cortex. The observed outcomes are in line with the previous cited reports accounting similar neurobehavioral, neuroinflammatory, neurochemical alterations following ICV-STZ infusion in mice. Although the exact etiopathology of SAD is still unclear however, it was demonstrated that cholinergic deficiency, elevated neurotoxic cytokines level, oxidative stress, augmented cholesterol synthesis are the considerable risk factors for development of SAD (Zameer et al., 2018). Numerous transgenic and non-transgenic animal models have been utilized to mimic the SAD like pathological events in order to assess the potential of new therapeutic targets (Lardenoije et al., 2018).

The proteolytic cleavage of APP by β and γ secretases in amyloidogenic pathway is the limiting step in cells for the generation of Aβ peptide. It has been known that β secretase compete with α secretase for APP proteolysis (Chow et al., 2010). In regard to this context, molecular docking study was carried out to gain insight into the ALN interaction with the active sites of target protein (BACE-1). It has been hypothesized that BACE-1 inhibitors should exhibit binding to catalytic sites in enzyme which could interrupt the interaction between enzyme and APP and therefore, prevent the cleavage of APP to Aβ peptide (Hassan et al., 2018). In in silico modelling, the number and nature of different bond types such as hydrogen bonds, hydrophobic bonds, and polar bonds play key role in ALN-BACE-1 interaction. The molecular docking revealed lower docking score in case of ALN comparing to other selected BACE-1 inhibitors. The lowest docking score interprets best binding conformation of ligand at its receptor site. This molecular docking study showing interaction between ALN and BACE-1 suggest that ALN could serve as molecule with multi-target for bone loss, modulation of cholesterol synthesis and cognitive functions.

ICV-STZ infused mice exhibited an impaired learning and memory performance as assessed by behavioral paradigms viz spontaneous alternation behavior (SAB) and Morris water maze (MWM) tests. Our results were in agreement with the previous findings that acknowledged devoid of learning and memory skills due to impairment in acquisition and retention in open field and MWM (Thome et al., 2018). Impaired cognitive abilities in mice after STZ infusion are triggered by exaggerated deposition of Aβ peptides in neurons by proteolytic cleavage of APP via BACE-1 enzyme which attributed to significant augmentation of neuroinflammation with oxidative stress. In addition to these, cholinergic deficit was also observed in STZ infused mice on the basis of increased ChEs activity. In the earlier report, it was shown that ALN

Table 2
Effect of ALN cholesterol level in ICV STZ induced AD in mice.

Groups	N	Treatment	Dose	Cholesterol level (μg/ml)
1	8	Water (Control)	10 ml/kg p.o.	107.6 ± 16.42
2	8	Citrate Buffer (Sham control)	5 μl i.c.v.	114.5 ± 21.31
3	8	Streptozotocin	3 mg/kg i.c.v.	133.6 ± 16.24
4	8	Streptozotocin + Alendronate	3 mg/kg i.c.v. + 1.76 mg/kg p.o.	108.9 ± 17.46
5	8	Streptozotocin + Donepezil	3 mg/kg i.c.v. + 5 mg/kg p.o.	110.6 ± 14.94
6	8	Alendronate per se	1.76 mg/kg p.o.	114.0 ± 13.26
7	8	Donepezil per se	5 mg/kg p.o.	111.8 ± 11.91

This table is showing the effect of STZ, ALN, DNP on cholesterol level (μg/ml) in mice hippocampus. No change was observed in cholesterol level in STZ infused mice as well as in drug treated groups. Each value is indicated as mean ± SEM. The differences between different groups were insignificant as given by one-way ANOVA followed by post-hoc Tukey-Kramer Multiple Comparison test.

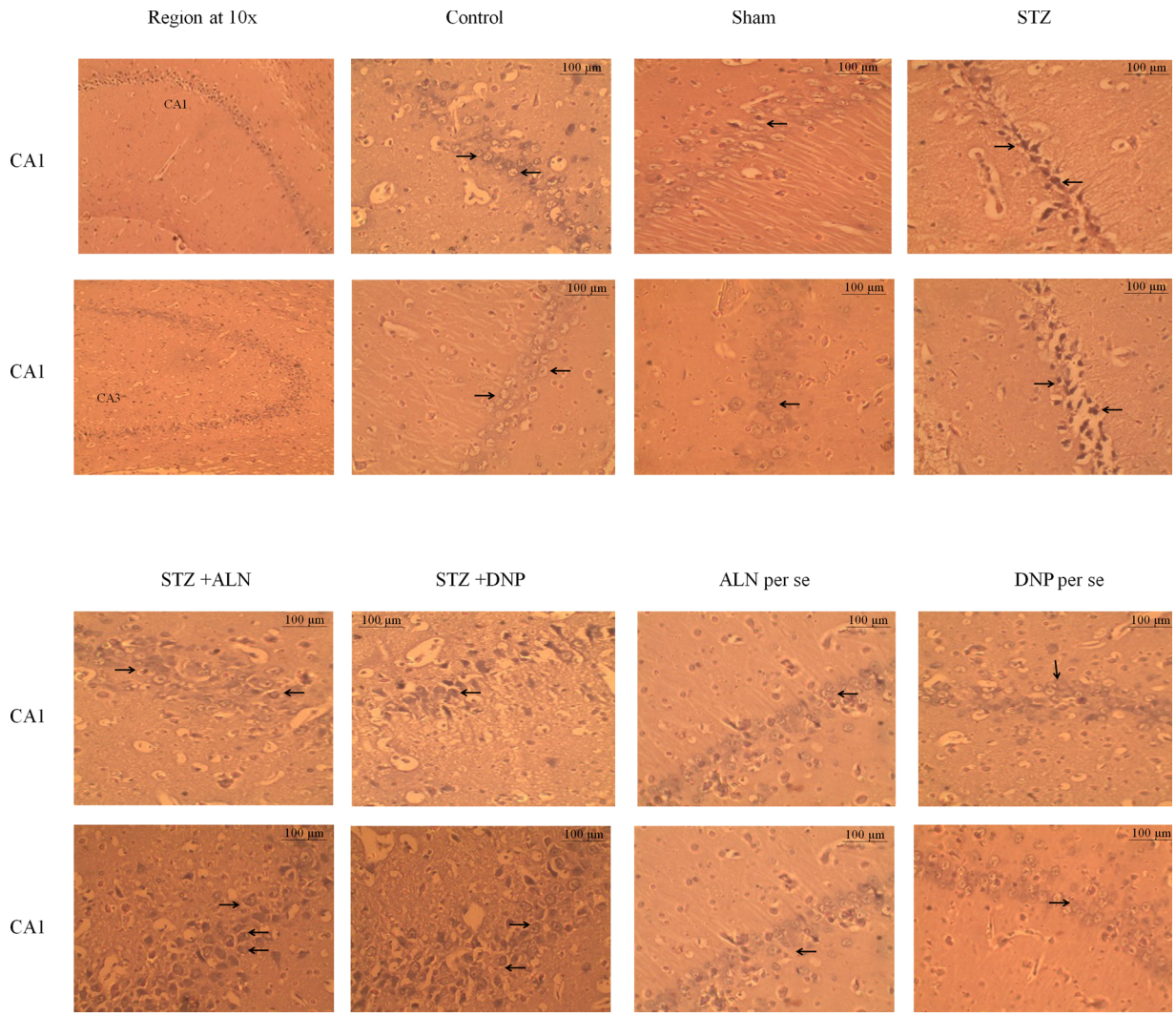

Fig. 9. Histopathological analysis.
Effect of ALN and DNP on ICV STZ induced histopathological alterations in CA1 and CA3 regions of mice hippocampal of groups; control, sham, STZ, STZ + ALN, STZ + DNP, ALN per se and DNP per se at 40x magnification using scale of 100 µm. ICV: Intracerebroventricular; STZ: Streptozotocin; ALN: Alendronate and DNP: Donepezil.

suppresses AChE level in frontal cortex of rat (Cibickova et al., 2007). In the present study, we investigated the plausible neuroprotective effect of alendronate on the cognitive skills in icv STZ treated mice. The effect of ALN on spatial memory was investigated by performing SAB test that depicted a significantly higher % alternation in STZ infused mice following oral administration of ALN. It was further confirmed by MWM test and the observed results indicated that ALN administration at a dose of 1.76 mg/kg p.o. daily during 15 consecutive days prevented diminished spatial learning and memory performance in STZ treated mice as indicated by the significant reduction in the escape latency and path length during spatial acquisition of MWM test. Akin to this observation, significant increase in percent dwell time by ALN was found during reference memory test in probe trial performed 24 h later to training session in MWM task. AD is supposed to be associated with impaired motor activity (Albers et al., 2015) that may also interfere with behavioral cognitive tasks, so we recorded the mean speed in zone in spatial acquisition trials in MWM in order to assess the effect on motor motor activity. No significant changes were noticed in mean speed in zone in mice of all groups. The severe impairment in performance of mice receiving ICV STZ in behavioral tasks coincides with the finding acknowledged that the deficit in MWM and SAB performance reflects neurobiological and neurochemical alterations underlying spatial memory modulation ((Yamini et al., 2018; Zameer and Vohora, 2017) Co-ordinated action of different brain regions mainly hippocampus and frontal cortex have been considered crucial for modulation of spatial and non-spatial memory (Arora and Deshmukh, 2017).

It is well-known that AChE is mainly responsible for degradation of ACh, a key neurotransmitter, into acetate and choline leading to impaired cholinergic neurotransmission responsible for cognitive decline (Agrawal et al., 2009). Over the several past years, AChE inhibitors responsible for increased synthesis of ACh have been highlighted to be one of the potential therapies in treatment of AD (Kumar et al., 2015; Ohta et al., 2017). In our experiment, ICV-STZ administration at subdiabetogenic dose caused significant augmentation of AChE activity in hippocampus and frontal cortex of mice brain. The normalization of increased AChE activity in hippocampus as well as frontal cortex seem to be the contributive cause of memory improvement following ALN administration in STZ infused mice. Our results were in support of finding of Cibickova and co-workers summarizing the inhibitory effect of ALN on AChE activity in different brain regions (Cibickova et al., 2007). Besides AChE inhibitors, agents inhibiting BChE enzyme has also gained importance in order to impart added benefits in modulation of learning and memory. BChE is a pseudocholinesterase enzyme contributes to approximately 20% hydrolysis of ACh and is acknowledged

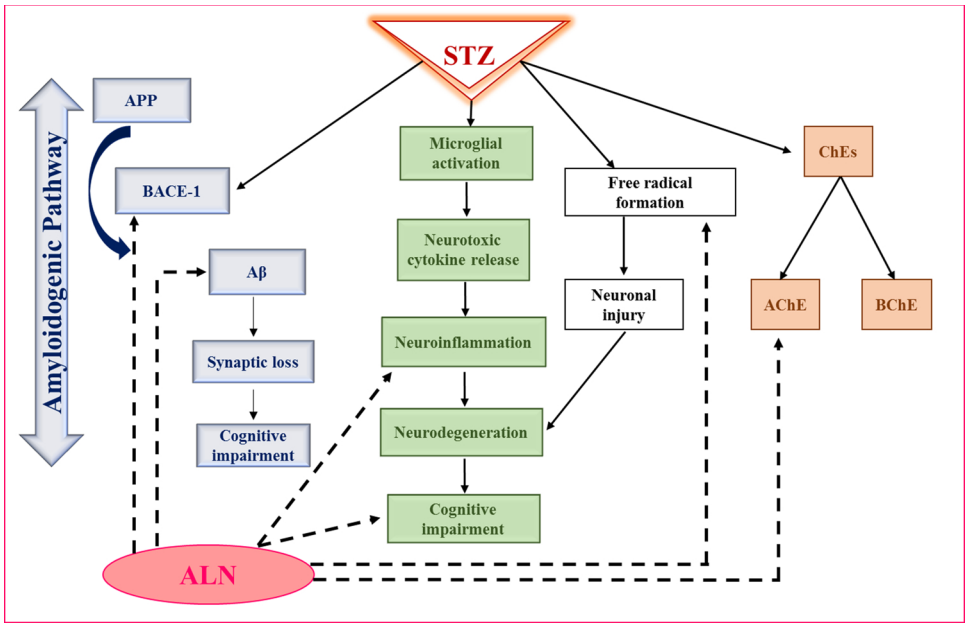

Fig. 10. Therapeutic implications.
ICV: Intracerebroventricular; STZ: Streptozotocin; ALN: Alendronate; SAB: Spontaneous Alternation Behavior; MWM: Morris Water Maze; ChEs: Cholinesterases; AChE: Acetylcholinesterase; BChE: Butylcholinesterase, Aβ: Amyloid Beta; BACE-1: β-site APP Cleaving Enzyme; TBARS: Thiobarbituric Acid Reactive Substances; SOD: Superoxide Dismutase and CAT: Catalase.

to be activated during AD in cortical and neocortical region (Costa et al., 2016; Kumar et al., 2015). Here in the current study, significant elevation in BChE activity was noted in mice belonging to ICV STZ group as compared to control and sham group. But no significant changes were observed in ALN and donepezil treated mice brain structure. Nevertheless, the significant rise in ChEs activities (both AChE and BChE) observed in mice hippocampus and frontal cortex induced by ICV STZ was similar to the cited report demonstrating memory deficit by STZ in mice due to increase in ChEs activities in different brain structures (Costa et al., 2016). But the mechanism underlying STZ induced enhancement in ChEs activity in brain is still unexplained that exposes it a subject of further research.

Amyloid hypothesis is one of the best scientifically supported etiologic hypothesis for AD stating that augmented collection of Aβ both intracellularly as well as extracellularly is the driving event in progression of neuronal damage, cognitive depletion and synaptic damage in this disease. The excessive production of Aβ in neurons occurs by the sequential proteolytic cleavage of β-amyloid precursor protein (APP) in amyloidogenic pathway through two enzymatic complexes viz beta- and gamma-secretase (Kametani and Hasegawa, 2018). In the current experiment of AD, level of $A\beta_{1\text{-}42}$ peptide, a neurotoxic form of Aβ and a major cause for senile plaques, was significantly increased in both hippocampus and frontal cortex following 14 days of ICV STZ infusion. Treatment with ALN and donepezil resulted in significant lower level of $A\beta_{1\text{-}42}$ in both brain regions. Corresponding to the above discussed molecular docking study and for better understanding, we estimated level of BACE-1 using ELISA kit in mice hippocampus and frontal cortex. We found increased level of BACE-1 enzyme in mice intracerebroventricularly injected with two alternative dose of STZ (3 mg/kg bilaterally) that coincides with the demonstration of El Halawany and colleagues study based on STZ model of SAD (Halawany et al., 2017). Significantly reduced activity of BACE-1 enzyme by alendronate treatment given for 15 days in STZ infused mice could therefore contribute to the attenuation in Aβ peptide accumulation in hippocampus and frontal cortex. Relevant to our finding it was recently demonstrated in rats that cholesterol modifying drugs, statins, have an ability to alleviate the diminished behavioral and neurological implications of subdiabetogenic ICV dose of STZ induced pathological events without relying on cholesterol synthesis (Dalla et al., 2010; Tramontina et al., 2011).

Neuroinflammation responses have been evidenced to contribute to the development of AD. Experimental data exist indicating that release of pro-inflammatory cytokines is related to imbalance formation and clearance of Aβ peptides in neurons (Pandey et al., 2018; Song et al., 2014). The augmented accumulation of Aβ is an attribute to brain damage that incite the overactivation of microglia which leads to detrimental effect on neuronal survival, release of neurotoxic cytokines and oxidative stress biomarkers (Block et al., 2007). Higher expression of cytokines viz., IL-6, IL1β and TNF-α has been coupled with in cognitive and cholinergic dysfunctions resembling age-linked AD and the same has also been acknowledged to occur in STZ model of SAD (Rai et al., 2013). In our experiment, we observed elevated release of IL-6, IL1β and TNF-α after toxicity development by subdiabetogenic ICV dose of STZ in mice. ALN and DNP treatment to ICV STZ infused mice were found to be able to significantly reduce these cytokines level in hippocampus as well as frontal cortex and the results were in same line as those found in in vitro and clinical studies (in serum) following ALN treatment (Cantatore et al., 1999; Giuliani et al., 1998). Moreover, the drastic cytokines release has also been investigated to be linked to free radical formation and mitochondrial dysfunction in neurodegenerative diseases (Cui et al., 2012). Corresponding to the finding of increased oxidative stress due to STZ injection in brain (Wang, Dongmei et al., 2018), we detected significantly higher level of TBARS, NO, and lower level of CAT and SOD. Generation of oxidants like free NO species was evidenced to inhibit normal cellular functioning in hippocampal neurons and ultimately leads to neuronal death. Here in the present experiment, ALN alleviated the subdiabetogenic ICV dose of STZ induced enhancement in oxidative stress markers (but not TBARS), indicating its anti-oxidant potential. Although, ample data is not known explaining the anti-oxidative and anti-inflammatory potential of ALN, however it has been shown to alter inflammatory cytokines and oxidant level in peripheral tissues and blood but no findings are available developing role of ALN against neuroinflammation and oxidative stress in brain.

Above all, STZ administration in mice brain in subdiabetogenic dose is supposed to mimic pathologies similar to SAD (Javed et al., 2013, 2015) but it has not been reported to alter cholesterol level in mice which is also a critical risk factor for development of SAD. However, in our study, the level of cholesterol was estimated in mice hippocampus of different groups through GC–MS technique to find out the effect of subdiabetogenic ICV dose of STZ on its level. No significant change in cholesterol level was found in STZ infused mice when compared with control and drug treated groups. Despite of being cholesterol lowering drug, ALN showed no influence on level of cholesterol in mice. Nonetheless, it was able to significantly alter other complications induced by

ICV STZ. Thus the outcomes noted against subdiabetogenic ICV dose of STZ induced changes in the current study could be linked to the potential of ALN to improve cognitive disabilities as well as neurobiological pathologies associated to SAD.

From the outcomes of current work, it could be concluded that ALN prevented impairment in learning and memory in a mouse model of SAD which might be attributable to the attenuation of neuroinflammation, oxidative stress and Aβ1-42 deposition prompted by ICV STZ infusion. This study presents a perspective on the interaction of Aβ and APP cleaving BACE-1 enzyme (β secretase) and neuroinflammation and the protective role of ALN. To the best of our knowledge, our study is the first to present the amileorative effect of ALN against subdiabetogenic ICV dose of STZ induced impairment in neurobehavioral, neuroinflammation, and neurochemical markers, rendering ALN a promising pharmacological intervention to prevent symptoms and slow down progression of SAD. The ensemble of our experimental findings discussed gives new insight to the mode of action of ALN that present the need of further investigation to elucidate the exact cellular and molecular mechanism which could elaborate the effect of ALN against pathological events similar to AD.

Conflict of interest

All the authors declare no competing financial interest in publishing this research work.

Acknowledgements

This study was supported by Sun Pharmaceutical Industries Ltd and Council of Scientific and Industrial Research (CSIR), India. The experimental work was carried out in the Neurobehavioral Pharmacology lab, Jamia Hamdard. The authors are thankful to Prof. Suhel Parvez for providing the necessary facilities of Department of Medical Elementology and Toxicology, School of Chemical and Life Sciences (SCLS), Jamia Hamdard, New Delhi.

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