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ORIGINAL PAPER

Drotaverine Inhibitor of PDE4: Reverses the Streptozotocin Induced Alzheimer's Disease in Mice

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease associated with decline in memory and cognitive impairments. Phosphodiesterase IV (PDE4) protein, an intracellular cAMP levels regulator, when inhibited act as potent neuroprotective agents by virtue of ceasing the activity of Pro-inflammatory mediators. The complexity of AD etiology has ever since compelled the researchers to discover multifunctional compounds to combat the AD and neurodegeneration. The aim of this study was to probe into role of drotaverine a PDE4 inhibitor in the management of AD. Albino mice were divided into seven groups (n = 10). Group 1 control group received carboxy methyl cellulose (CMC 1 mL/kg), group II diseased group treated with streptozotocin (STZ 3 mg/kg) by intracerebroventricular (ICV) route, group III administered standard drug Piracetam 200 mg/kg and groups IV–VII were given drotaverine (10, 20, 40, and 80 mg/kg i/p respectively). Groups II–VII were given STZ (3 mg/kg, ICV) on 1st and 3rd day of treatment to induce AD. All the groups were given their respective treatments for 23 days. Improvement in learning and memory was evaluated by using behavioral tests like open field test, elevated plus maze test, Morris water maze test and passive avoidance test. Furthermore, brain levels of biochemical markers of oxidative stress, neurotransmitters, β-amyloid and tau protein were also measured. Drotaverine showed statistically significant dose dependent improvement in behavioral and biochemical markers of AD: the maximum response was achieved at a dose level of 80 mg/kg. The Study concluded that drotaverine ameliorates cognitive impairment and as well as exhibited modulated the brain levels of neurotransmitters.

Keywords STZ · Drotaverine · Cognitive impairments · Neurotransmitters · Alzheime

Abbreviations

Aβ	Amyloid beta
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
BSA	Bovine serum albumin
BT	Brain tissue
cAMP	Cyclic adenosine monophosphate

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CAT	Catalase
CMC	Carboxymethyl cellulose
EPMT	Elevated plus maze test
GSH	Glutathione
ICV	Intracerebroventricular
IL	Initial latency
MWMT	Morris water maze test
SAC	Sacrificed
STZ	Streptozotocin
SOD	Superoxide dismutase
TCA	Tricholoro acetic acid
TBA	Thiobarbituric acid
TL	Transfer latency

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder which affects hippocampus and neocortical regions of the brain resulting in behavioral and memory impairments [1, 2], which interferes with routine activities of the subject [3]. AD mostly occurs at old age and its onset before 65 years of age is less than 10% [3]. Globally its prevalence is about 33.9 million and it's assumed that the figure will be tripled after 40 years. Epidemiological studies showed that 5.3 million people in the US suffered with memory impairment [4]. The most common and acceptable method for the induction of AD is injecting the streptozotocin (STZ) through intracerebroventricular route (ICV) [5], which interfere with glucose metabolism in the brain by inhibiting insulin receptors, along with long term memory and learning loss [6] as well as neuroinflammation, ROS (Reactive oxygen species) generation, neuronal injury and neurodegeneration that lead to the formation of amyloid beta plaques and aggregates of tau protein in brain. ICV injection of STZ showed glucose hypo-metabolism, cholinergic deficits, apoptotic cell death, neurodegeneration and finally cognitive impairment [7]. Previous study reported that ICV-STZ administration at dose level of 3 mg/kg causes the sporadic Alzheimer and increase the all parameters that are involved in the pathogenesis of AD [5, 8]. Neuropathological findings of Alzheimer's disease include accumulation of amyloid beta peptides and tau containing neurofibrillary tangles [9, 10]. Amyloid beta protein gets accumulated around meningeal, cerebral vessels and in the gray matter. Neurofibrillary tangles of tau protein are initially found in hippocampus and then throughout the cerebral cortex [3]. Phosphodiesterases are enzymes which are used to set the intracellular levels of the second messengers (cAMP and cGMP) by putting check on their rate of breakdown. Phosphodiesterase IV (PDE4) is encoded by 4 genes i.e. PDE4A-PDE4D. PDE4A, B and D are expressed in hippocampus, hypothalamus, cerebellum, cortex and striatum while PDE4C is hardly expressed in brain [11]. PDE4 were well established for memory enhancing, anti-inflammatory and anti-depressant along with smooth muscle relaxation [12]. It acted through elevation of cAMP (Cyclic adenosine monophosphate) levels which result in formation of cAMP adenyl cyclase enzyme. cAMP switches on the protein kinase A which in turn phosphorylates the cAMP response element binding protein (CREB). CREB triggers transcription of genes which are associated with cognitive improvement by increasing synaptic plasticity (increase in strength of neurons) and neurogenesis [11]. Drotaverine, being selective inhibitor of PDE isoenzyme IV [13] is said to be involved in cognitive improvement similar to PDE II inhibitors by enhancing cAMP signaling [14]. Previous studies on PDE4 inhibitor, rolipram, revealed cognitive improvement and anti-depressant effects by inhibiting the cAMP degradation in brain [11, 15]. Due to ever increasing prevalence of AD and previously reported PDE4 inhibitor potential in AD, it is worthwhile to investigate the potential of drotaverine as a modulator of STZ induced memory impairment by exerting its action on multiple targets including reduction in acetylcholine esterase (AchE) level as well as elevation of neurotransmitters levels in brain. The aim of this study is to find out the effect of drotaverine in memory restoration process and in AD. It is expected that drotaverine may provide the rationale approach for the treatment of the AD by combating the memory deficit and neuroinflammation and might prove therapeutically useful agents for AD.

Material and Methods

Drugs and Chemicals

Drotaverine (Searle pharmaceutical), Piracetam (GSK laboratories), Streptozotocin, Hydrogen peroxide, Sodium hydroxide, Pyrogallol, di-sodium Hydrogen phosphate, Isoflurane and Serotonin (Sigma-Aldrich), Hydrochloric acid, Magnesium chloride, Calcium chloride, Dextrose, Folin-Ciocalteus's (Merck), DTNB (5,5′-dithiobis-(2-nitrobenzoic acid) (Ark pharm) were purchased. Sodium phosphate monobasic, potassium phosphate monobasic was obtained from Riedel-de-Haen USA. Dopamine (Haji medicine Pakistan) and nor-Adrenaline (Norepine) (Ontech Corporation China) were purchased respectively.

Experimental Animals

Swiss albino adult mice weighing 25–40 g, 6–8 weeks old of both sex (male & female) were purchased from Riphah International University, Lahore Pakistan. All animals were kept under standard lab conditions i.e. 12:12 h light: dark cycle, temperature 25 ± 1 ℃ and humidity 45–55%. Mice

were allowed easy access to diet and water. All the experiments were carried out between 8:00 pm to 4:00am.

Ethical Approval

All methods were approved by research ethical committee of Riphah International University, Lahore with an authorized number of REC/RIPS-LHR/011 ruled under the regulation of National Institute of Health Guide for the Care and Use of Laboratory Animals (1996).

Experimental Design

Animals divided into seven groups containing 10 mice. Group 1: Vehicle control group received CMC (Carboxy methyl cellulose) 1 mL/kg i.p; Group II STZ group 3 mg/kg i.p; Group III: Piracetam (Positive Control) 200 mg/kg i.p; Group IV: 10 mg/kg; Group V: 20 mg/kg; Group VI: 40 mg/kg; Group VII: 80 mg/kg of drotaverine respectively through intraperitoneal route. STZ 3 mg/kg ICV unilaterally [16] was given to all groups except control group, at 1st and 3rd day of experiment by using stereotaxic apparatus. All other treatments were given for 23 days (Fig. 2.1). The doses of drotaverine are selected on the basis of human doses using conversion formula given by Nair et al. [17]. Doses were administered to animals according to the weight of experimental animals [17]. Morbid sign and symptoms and mortality were checked daily.

Behavioral Tests

Open Field Test

Open field apparatus was used for the open field test consisted of area (square) 40 cm × 40 cm and walls with height of 36 cm. The square area is divided into 16 sub-squares. Central area is a region consisting of four sub squares marked with red color. Each mouse was put in the centre at start of test. The activity of each mouse was recorded for 300 s [18]. Mouse was immediately move towards periphery (marked as Blue) and the time to move from centre to periphery was recorded which is called latency time [19]. In this test number of crossings and time spent in the centre and periphery were recorded. Other parameters recorded in open field test were rearing, fecal pallets, time of immobility, jumping and efforts made by each mouse to getaway were observed [20].

Elevated Plus Maze Test (EPMT)

A test for the exploration of spatial memory is elevated plus maze test. Apparatus consisted of two open and two closed arms. The height of the apparatus was 25 cm from floor [21]. Two open arms positioned opposite to each other measured (15 cm × 5 cm) while closed arms, positioned like open arms measured (15 cm × 5 cm × 16 cm) and central platform was (5 cm × 5 cm). Closed arms have side walls (16 cm height) while open arms have no side walls and open from all sides. EPMT performed on 13th and 14th day of treatment. In EPMT, behavior of mouse was recorded for 300 s [22]. Mouse was placed at end of the open arm facing away from central platform and transfer latency was measured which a time is taken by the mouse to move into any one of the closed arm with all the four paws. If mouse did not enter into closed arm within 90 s, it was gently pushed in any one of the closed arm and transfer latency was taken as 90 s. Memory retention was measured after 24 h [23]. Number of entries and time spent in either of the arms was also calculated in elevated plus maze test [22].

Morris Water Maze Test

Water Morris test was used to evaluate the spatial memory and learning. It contains circular tank having 150 cm in diameter and height of 60 cm. Water filled upto 40 cm. Temperature of water was maintained at 23 ± 1 °C. Water maze consists of four quadrants i.e. North, South, East, and West by evenly or uniformly spaced locations on periphery. Water was made opaque by adding white, non-toxic paint (calcium hydroxide). Pool consists of platform which is 10 cm in diameter and platform will be placed in the center of any one of the quadrant [2]. Morris water maze test (MWMT) was performed on 15th, 16th, 17th and 18th (acquisition days) and on 19th (probe test) day of drug treatment. During training periods animals find the hidden platform and maximum 60 s time was given to animal for finding the platform. Animals are allowed to stay on it for 30 s before next trial. If animal failed to find the hidden platform within 60 s, the mouse was put gently on platform during training session [24]. After training, probe test was conducted without platform and the mouse was observed for 180 s. Escape latency (time taken by the animal to move from starting point to the target quadrant in order to find hidden platform), time spent and number of crossings were recorded in the quadrant in which platform had been placed before during training [25].

Passive Avoidance Test

Passive avoidance test is a fear aggravated test in rodent model for CNS disorders and used for evaluation of learning and memory. Apparatus consisted of a box (20 cm × 20 cm × 20 cm) having four walls with a wooden platform (10 cm × 7 cm × 1.7 cm) in the center [23]. Passive avoidance test was performed on 22nd and 23rd day. In this test, each mouse was put on platform and step down latency was measured, later known as initial latency to step down,

then immediately through stainless steel grid floor, electric foot shock of (3 s, 0.4 mA) was given to animal. After series of trials mouse remained on the platform. Retention time was performed after 24 h without electric shocks. Retention latency was noted in the same manner as previously recorded in training session. The cut off time was 300 s [2].

Measurement of Biochemical Parameters

Preparation of Brain Homogenate

All of the treated animals of each protocol were anesthetized by using 3 to 5% isoflurane with oxygen on the 24th day of treatment, i.e. after the completion of all behavioral and locomotors assessments. Brains were taken after scarification and rinsed with cold normal saline (0.9% NaCl). Tissue homogenates were prepared followed by addition of 0.1 M phosphate buffer (7.4), in 1:10 ratio. Centrifuge the homogenate at 6000 rpm ± 4 ℃ for 10 min. Supernatant were collected for the performance of the different biochemical and Elisa assays.

Biochemical Analysis

Glutathione Level Estimation In tissue homogenate (1 mL), 1 mL of 10% trichloroacetic acid was added for protein precipitation and in supernatant added phosphate solution (4 mL) and 0.5 mL of 5,5-dithiobis-2-nitrobenzoic acid.

(DTNB) reagent and absorbance was observed at 412 nm. GSH level expressed in µg or reduced glutathione per mg of protein [26]. Following formula was used for the calculation of GSH levels:

$$\text{GSH level} = Y - 0.00314 \div 0.0314 \times DF \div Bt \times Vu,$$

Y is absorbance taken at 412 nm, B_T is brain tissue homogenate, D_F is dilution factor and V_U is aliquot volume (1 mL).

Measurement of Catalase Activity The mixture consisted of 0.05 mL of supernatant of tissue homogenate and 1.95 mL of 50 mM of phosphate buffer pH 7.0. 1 mL of 30 mM hydrogen peroxide (H_2O_2) was added to the mixture and change in absorbance at 240 nm was measured. It's values were expressed as micromoles of H_2O_2 oxidized per minute per milligram protein [26].

Catalase activity was measured by using following formula:

$$\text{Catalase activity} = \text{O.D.} \div E \times \text{Vol of sample} \times \text{mg of protein}$$

where δ O.D. is the change in absorbance per minute; E is extinction coefficient (0.071 $\text{mmol}^4\ \text{cm}^{-1}$) of hydrogen peroxide.

Estimation of Superoxide Dismutase (SOD)

Brain SOD levels were determined by using following method. A 3 mL mixture containing about 2.8 mL of potassium phosphate buffer (0.1 M, pH 7.4), 0.1 mL tissue homogenate and 0.1 mL of pyrogallol solution was prepared. Pyrogallol is known as auto-oxidizer agent that works in the alkaline solution and generating the free oxygen molecules. Super oxide dismutase enzyme rapidly reduced the oxygen the superoxide anion radicals. The absorbance of the resultant mixture was measured at 325 nm using UV spectrophotometer [18, 27, 28].

SOD contents were determined using the following regression line equation.

$$Y = 0.0095x + 0.1939$$

Measurement of Malnodialdehyde (MDA)

In supernatant (1 mL) and TBA (Thiobarbituric acid, 4.0 mM) reagent (1 mL) were dissolved in 100 mL of glacial acetic acid reagent. 3 mL of mixture was shaked and set aside for 15 min, followed by cooling. The mixture was centrifuged at 3500×*g* for 10 min. Absorbance of supernatant was measured at 532 nm and results was expressed as micromoles/mg of protein [26].

Quantification of MDA was done by using following formula:

$$\text{Conc. of MDA} = \text{Abs}_{532} \times 100 \times Vt \div (1.56 \times 105) \times Wt \times Vu,$$

where Abs_{532} is absorbance, V_T is volume of mixture (4 mL), 1.56×10^5 is the molar extinction coefficient, W_T is dissected brain's weight, V_U is aliquot volume (1 mL).

Estimation of Nitrite

Nitrite level was determined with spectrophotometer using Griess reagent. Equal quantity of brain homogenate and Griess reagent were mixed. The mixture was incubated for 10 min and the absorbance was observed at 546 nm [29]. Following regression line equation of sodium nitrite was used for determination of nitrite level.

$$Y = 0.003432x + 0.0366$$

Evaluation of Protein Content

Protein Contents were determined according to the method (Lowery et al. 1951) and values were expressed in µg/mg. Three solutions were prepared A. 2% Na_2CO_3 in 0.1 N NaOH; B. 1% NaK Tartarate in H_2O; C. 0.5% $CuSO_4 \cdot 5H_2O$ in water Reagent 1 was prepared by mixing 48 mL of A

soln,1 mL of B soln and 1 mL of soln. C Reagent 2 contained 2:1 part 1 part Folin-phenol [2 N]:1 part H_2O.

For determination of protein contents tissue homogenate (0.2 mL), 4.5 mL of Reagent 1 was added and mixture was incubated for 10 min then Reagent 2 (0.5 mL) was added to the mixture and incubated for 30 min [30]. The absorbance of the resultant mixture was measured at 660 nm. Following regression line equation of BSA was used to determine the protein contents.

$$Y = 0.00007571x + 0.0000476$$

Estimation of Acetylcholinesterase Activity

Acetyl cholinesterase activity was estimated by Elleman's method. To 0.4 mL of tissue homogenate added 2.6 mL of phosphate buffer (0.1 M with pH 8) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) about 100 µL and absorbance was noted (basal reading) at 412 nm, then to the solution substrate of acetylthiocholineiodide (20 µL) was added and change in absorbance was measured for 10 min at 2 min interval. Change in absorbance per minute was determined by using following formula and acetyl cholinesterase activity was expressed in µM/L/min/g of tissue [31].

$$R = 5.74(10^{-4}) \times \Delta A/co,$$

where R is the rate, in moles of substrate which was hydrolyzed/min/g of tissue. ΔA is the change in the absorbance per min. co is the original concentration of tissue expressed in mg/mL.

Estimation of Neurotransmitters

Preparation of Aqueous Phase

Tissue homogenate was prepared in HCl-butanol (5 mL) and centrifugation the homogenate at2000 rpm for 10 min. To aliquot of supernatant (1 mL) added heptane (2.5 mL) and 0.31 mL HCl (0.1 M) then shook it vigorously and centrifuged for 10 min at 2000 rpm for the separation of two phases. All the experiments were carried out at 0 ℃. Organic phase was discarded and aqueous phase (0.2 mL) was used for estimation of nor adrenaline, serotonin and dopamine.

Estimation of Serotonin Level

To 0.2 mL of aqueous phase 0.25 mL of o-phthaldialdehyde (OPT) was added and heated the mixture at 100 ℃ for about 10 min. Absorbance was noted at 440 nm after the temperature of sample reached to ambient temperature. 0.25 mL of HCl without adding OPT was used as blank [32]. Serotonin level was determined by using regression line equation of serotonin.

$$Y = 0.00314x + 0.1067$$

Estimation of Dopamine and Nor Adrenaline Level

To 0.2 mL of aqueous phase, added 0.05 mL of HCl (0.4 M) and 0.1 mL of ethylenediaminetetraacetic acid (EDTA)/sodium acetate (pH 6.9). Then 0.1 mL of iodine solution (0.1 M in ethanol) was added for oxidation process. Oxidation reaction was stopped by addition of Sodium sulphite (Na_2SO_3, 0.1 mL) then added in it 0.1 mL of acetic acid after 1.5 min and heated at 100℃ for 6 min. Allow the sample to cool at the room temperature. Absorbance of dopamine (at 350 nm) and nor-adrenaline (at 450 nm) was measured. Blank for dopamine and nor adrenaline was prepared by addition of respective reagents of oxidation in reverse order i.e. Na_2SO_3 before iodine [32]. Levels were calculated by using regression line equation of dopamine and nor adrenaline.

(Dopamine) $Y = 0.2331x + 0.0164$ (Nor adrenaline) $Y = 0.1008x + 0.2508$

In-Silico Modelling

The in-silico experiment was performed to investigate the activity of drotaverine for Acetyl cholinesterase (AChE) by the function of induced fit docking in Molecular Operating Environment (MOE) 2015.10. The three dimensional (3D) conformers of Piracetam (CID: 4843) and Drotaverine (CID: 1712095) were retrieved from PubChem database and further converted and optimized into PDB formats in Accelrys discovery studio visualize v17.2. The 3D X-ray crystallized structure of AChE (PDB ID: 4EY6) was retrieved from RSCB Protein Data Bank (http://www.rscb.org/). These structures were prepared in Structure Preparation application of MOE preceding the induced fit docking simulation. The macromolecule was inspected and optimized to address the structural problems such as in alternates, termini, H-counts and geometrical constraints of residues. Protonate 3D application was used to optimize the residues with rotamer, protomer and tautomer states. Following the protonation state and partial charge calculation, the ligands and macromolecule were further energy minimized with Charmm27 forcefield to refine and relieve the geometrical constraints and bad crystallographic contacts. Binding site, for ligand's docking, was identified by the specifying the pocket atoms for co-crystallized ligand. Dock application was used to setup the induced fit docking protocol using MOE dock panel. Ligands were docked into receptor's binding site by Triangle Matcher placement method using London dG scoring function that followed induced fit refinement

using GBVI/WSA dG scoring function to re-score the 30 poses. The pose with lowest conformational energy (i.e. S) was further used to investigate the spatial configuration and interactions between the ligand and receptor using Accelrys discovery studio visualizer v17.2. The docking protocol was validated by the cognate re-docking of co-crystallized ligand of macromolecule.

Protein Analysis by ELISA

Elisa kits were used for the estimation of the Amyloid beta ad tau proteins. Aβ 1–40 and tau proteins were complexes with HRP conjugates after that TBM solution was added. Reaction was stopped with the help of stopping solution. Change in color from blue to yellow was observed at 450 nm. Estimated proteins were quantified by using their respective standard curve [33].

The B_{1-40} Amyloid levels (pg/mL) were calculated by following regression line equation:

$$y = 0.00397(x) + 0.1504$$

The Tau levels in pg/mL were calculated by following regression line equation:

$$y = 0.0008508(x) + 0.7008$$

Histopathological Studies

Brain tissue was removed by sacrificing and preserved in 10% formalin solution. Fixation was carried out in paraffin wax blocks. Sections of tissue were cut at 40 µm by using the digital microtome. For qualitative Histopathological analysis de Olmos silver stain was used. 100X magnification power were used for the Histopathological analysis of the cutting sections of brain.

AChE Analysis Through RT-PCR

Tissue of brain was homogenized and treated with triazole solution and RNA was extracted. Quantitect reverse transcription kit was used for the transcription of RNA to cDNA. After that PCR studies were carried out under following conditions: 95 ℃ for 5 min followed by 40 cycles, annealing temperature is 60 ℃, further extended to 72 ℃ for 20 s. Primers list are given in a Table 1.

mRNA expression of AChE levels was detected by real time PCR and GADPH was used as internal control. Image J software was used to quantify the mRNA expression [34].

Statistical Analysis

All results were expressed as mean ± SEM. Data were analyzed by using the Graph pad prism software version 5.01(USA). One-way ANOVA followed by Dunnett's t-test and two-way ANOVA followed by Bonferroni's multiple comparison test was employed to analyze the statistical significance between the groups. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$was considered as mild, moderate and high level of significance.

Results

Behavioral Observations

Effect of Drotaverine on Cognitive Improvement Using Open Field Test

In open field test, drotaverine at dose level of 80 mg/kg showed significant ($P < 0.05$) decrease in the latency when compared to the streptozotocin-treated group and positive control group. Animals showed dose-dependent increase in locomotor activity i.e., by decreasing the dose, freezing time was decreased while rise in the number of crossings were observed. Animals spent more time in the central arena; as compared to periphery: the time being significantly ($P < 0.05$) enhanced when compared with selected groups of animals as shown in footnote of the Table 2.

Effect of Drotaverine in Cognitive Improvement Assessed by Using Elevated Plus Maze Test

Drotaverine showed dose dependent decrease in the latency time as the dose increases the time to enter in the open arm was also increased when compared the trial day to test day (Fig. 1). No. of open arm entries were also significantly increased by drotaverine when compared to STZ group (Table 3).

Table 1 Primers list

Primer	Sequence	
Acetylcholinesterase (AChE)	Forward AGGACGAGGGCTCCTACTTT	Reverse CATGGCATCTCTCAGGTGGG
Glyceraldehyde 3-phosphate dehydrogenase (GADPH)	Forward GGAGTCCCCATCCCAACTCA	Reverse GCCCATAACCCCCACAACAC

Table 2 Effect of drotaverine in cognitive improvement using open field apparatus

Treatment groups	Latency (s)	Rearing (no.)	Freezing time (s)	No. of crossings	Time spent		Defecation
					Periphery (s)	Centre (s)	
Control	3 ± 0.3	9.4 ± 0.4	70 ± 1.2	38.5 ± 1.3	121.8 ± 1.5	7.1 ± 0.6	No
STZ	6.3 ± 0.8$^{\#}$	2.8 ± 0.2$^{\#}$	155 ± 1.5$^{\#}$	15.5 ± 0.5$^{\#}$	160 ± 1.3$^{\#}$	2.5 ± 0.3$^{\#}$	Yes
Piracetam (200 mg/kg)	2.1 ± 0.2*	6 ± 0.5	80 ± 0.5*	27.5 ± 1.4	120.8 ± 1.4	6.6 ± 0.7*	No
Drotaverine (10 mg/kg)	4.8 ± 0.8	4.2 ± 0.2	132 ± 1.3*	17.8 ± 1.2	156 ± 0.7	3 ± 0.5	Yes
Drotaverine (20 mg/kg)	3.5 ± 0.4*	10 ± 1.0	100 ± 0.7*	30.0 ± 0.9	145 ± 0.4	5 ± 0.2*	Yes
Drotaverine (40 mg/kg)	2.3 ± 0.2*	13.6 ± 1.3*	78 ± 1.2*	37 ± 1.2*	109 ± 0.7*	7.5 ± 0.5*	No
Drotaverine (80 mg/kg)	1.1 ± 0.1$^{*,\#,\alpha}$	15 ± 2.2$^{*,\#,\alpha}$	50 ± 1.4$^{*\#,\alpha}$	48 ± 1.3$^{*,\alpha}$	99.0 ± 1.4$^{*,\#}$	9.3 ± 0.7$^{*,\#,\alpha}$	No

Data are presented as mean ± SEM, n = 10

*P < 0.05 when compared with Streptozotocin-treated group

$^{\#}$P < 0.05 in comparison to control and $^{\alpha}$P < 0.05 in comparison to positive control

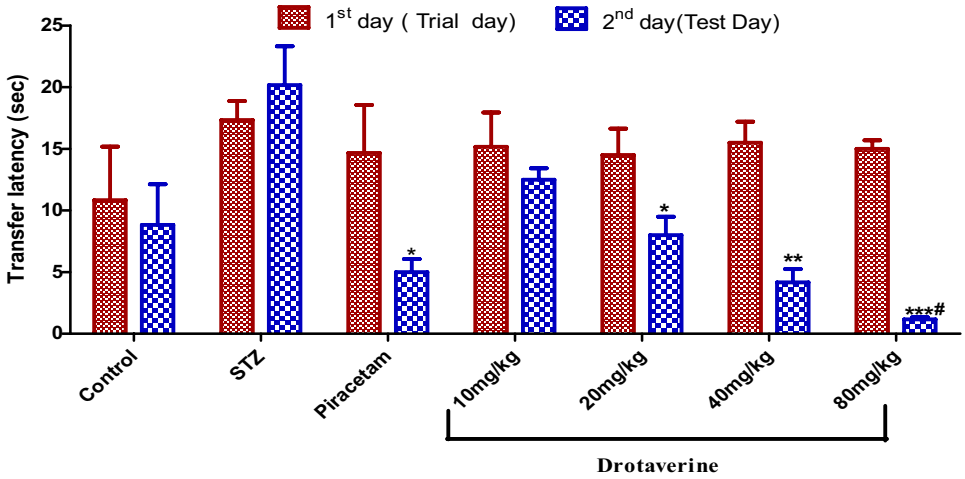


Fig. 1 Effect of drotaverine at different dose levels on transfer latency (s) in elevated plus maze test. Data are presented as mean ± SEM, n = 10. *P < 0.05, **P < 0.01 and ***P < 0.001 when given when compared with STZ group. $^{\#}$P < 0.05 in comparison to control group

Table 3 Number of entries in open and closed arms of elevated plus maze test

Treatment groups	No. of entries in open arm	No. of entries in closed arm
Control	2.1 ± 0.2	1.8 ± 1.0
STZ	1.2 ± 0.7$^{\#}$	3.1 ± 1.5$^{\#}$
Piracetam (200 mg/kg)	2.0 ± 0.3*	2.1 ± 1.2*
Drotaverine (10 mg/kg)	1.4 ± 0.2*	2.6 ± 1.2$^{*,\#}$
Drotaverine (20 mg/kg)	1.6 ± 1.0*	2.5 ± 0.7$^{*,\#}$
Drotaverine (40 mg/kg)	1.84 ± 0.4*	2.3 ± 1.0*
Drotaverine (80 mg/kg)	2.2 ± 0.9*	1.6 ± 0.6*

Each numerical value indicates mean ± SEM of 10 animals

*P < 0.05 when compared with streptozotocin-treated group

$^{\#}$P < 0.05 vs control

Effect of Drotaverine in Cognitive Improvement Measured by Morris Water Maze Test

Drotaverine at 80 mg/kg dose caused highly significant (P < 0.001) reduction of escape latency when compared with streptozotocin-treated group (Fig. 2). The drug at the same dose level showed significally (P < 0.05) increase in the number of crossings and time spent in north quadrant when compared with values of STZ-treated group (Figs. 3, 4). STZ group showed increase in escape latency at both days (Table 4).

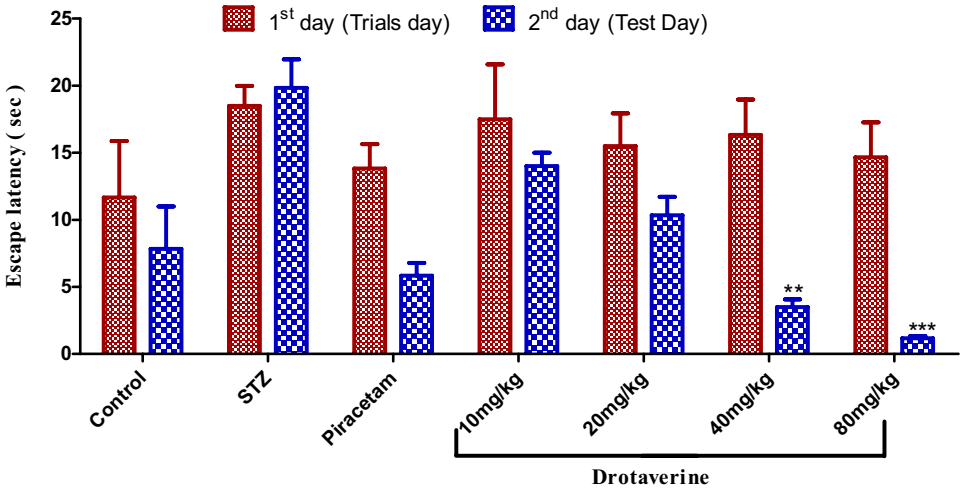


Fig. 2 Effect of drotaverine at different dose levels on escape latency (s) in Morris water maze test. Data are presented as mean ± SEM, n = 10. **P < 0.01 and ***P < 0.001 was given when compared with 1st day and Streptozotocin treated group

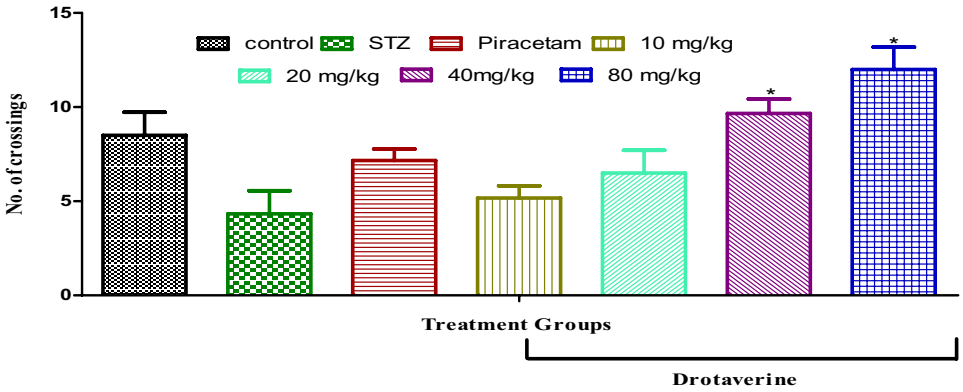


Fig. 3 Effect of drotaverine at different dose levels on no. of crossings in Morris water maze test. Data are presented as mean ± SEM, n = 10. *P < 0.05 when compared with streptozotocin-treated group

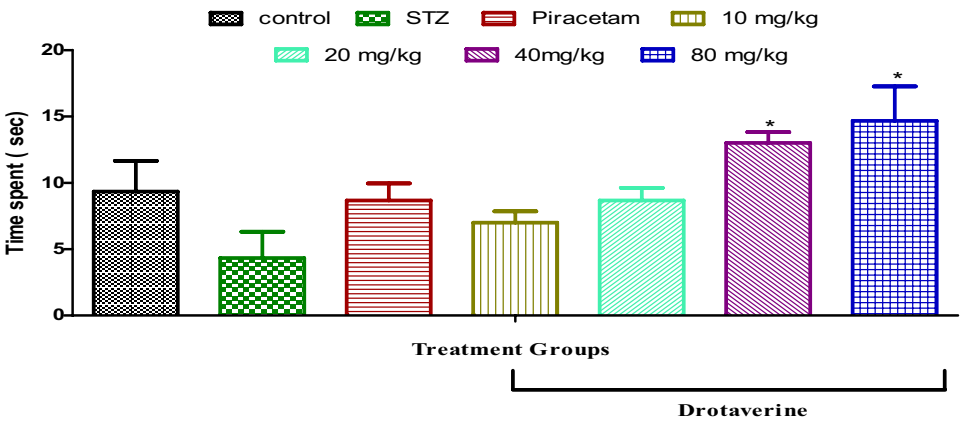


Fig. 4 Effect of drotaverine at different dose levels on time spent (s) in Morris water maze test. Data are presented as mean ± SEM, n = 10. *P < 0.05 when compared with streptozotocin treated group

Effect of Drotaverine in Cognitive Improvement by Applying Passive Avoidance Task

Retention latency time was increased in all treatment groups except streptozotocin-treated group on test day after chronic dosing for 23 days. The increase in retention latency time was highly significant caused by drotaverine at all doses when test day values were compared 1 day preceding training values (Fig. 5).

Table 4 Time spent in open and closed arms of elevated plus maze test

Treatment groups	Time spent in open arm (s)	Time spent in closed arm (s)
Control	9.4 ± 1.5	151 ± 1.0
STZ	5.0 ± 1.2$^{\#}$	190 ± 1.8$^{\#}$
Piracetam (200 mg/kg)	8.6 ± 0.9*	154 ± 1.2*
Drotaverine (10 mg/kg)	6.1 ± 1.0*	171 ± 0.5*
Drotaverine (20 mg/kg)	7.3 ± 0.4*	163 ± 1.7*
Drotaverine (40 mg/kg)	8.4 ± 1.2*	145 ± 1.2*
Drotaverine (80 mg/kg)	9.3 ± 0.7*	135 ± 0.9*

Data are presented as mean ± SEM, n = 10

*P < 0.05 when compared with streptozoocin-treated group

$^{\#}$P < 0.05 vs control

Morbidity Sign and Symptoms

No morbidity and mortality were observed during the whole study period.

Effect of Drotaverine at Different Dose Levels on Biochemical Markers

Endogenous antioxidant levels (SOD, CAT & GSH) were significantly (P < 0.05) rose in the brain of animals treated with the drotaverine at different dose levels when compared to STZ group. Drotaverine at 80 mg/kg dose levels increased the endogenous antioxidants levels more than normal (Table 5). while STZ group showed significant decrease antioxidant level. Though drotaverine at all dose levels increased the MDA levels which did not achieve the significant (P > 0.05) levels when compared to streptozotocin-treated group. Drotaverine at dose of 80 mg/kg showed significally (P < 0.05) decreased the nitrite level as compared to STZ-treated group. Surprinsingly drotaverine at dose level 20 mg/kg showed reduction in nitrite level whose magnitude was similar to that achieved by piracetam group treatment (Table 5). AChE levels were significantly decreased with the treatment of drotaverine indicating the increased levels of acetylcholine in the brain that involved in the memory and learning process.

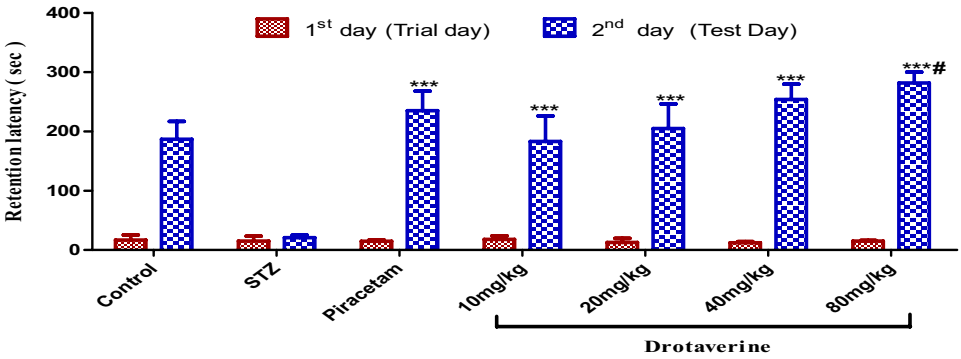


Fig. 5 Effect of drotaverine treatment at different dose levels on retention latency (s) in passive avoidance task. Data are presented as mean ± SEM, n = 10. ***P < 0.001 when compared with streptozotocin-treated group. $^{\#}$P < 0.05 in comparison to control group

Table 5 Effect of drotaverine at different dose levels on biochemical assays

Treatment groups	GSH (µg/mg of protein)	CAT (µmol/min/mg of protein)	SOD (µg/mg of protein)	MDA (µmol/mg of protein)	Nitrite (µg/mg of protein)	Protein (µg/mg of protein)	AChE (µmol/min/mg of protein)
Control	25 ± 0.1	150 ± 0.5	43.0 ± 0.1	0.16 ± 0.8	3.3 ± 0.03	460 ± 0.8	1.5 ± 0.04
STZ	20 ± 0.03$^{\#}$	96.1 ± 0.5$^{\#}$	24.7 ± 0.2$^{\#}$	0.22 ± 0.5$^{\#}$	4.8 ± 0.05$^{\#}$	390 ± 1.7$^{\#}$	2.3 ± 0.2$^{\#}$
Piracetam (200 mg/kg)	22 ± 0.1*	114 ± 0.3*	41 ± 0.1*	0.17 ± 1.2*	3.7 ± 0.03*	463 ± 1.4*	1.4 ± 0.7*
Drotaverine (10 mg/kg)	21 ± 0.1	107 ± 0.3*	28 ± 0.1*	0.39 ± 0.8$^{\#\alpha}$	3.9 ± 0.06*	442 ± 0.8*	1.6 ± 0.1*
Drotaverine (20 mg/kg)	24 ± 0.1*	115 ± 0.6*	35.7 ± 0.2*	0.37 ± 0.5$^{\#\alpha}$	3.7 ± 0.05*	480 ± 2*	1.5 ± 0.02*
Drotaverine (40 mg/kg)	25 ± 0.05*	121 ± 0.4*	38.4 ± 0.3*	0.38 ± 0.5$^{\#\alpha}$	3.6 ± 0.03*	626 ± 2.3*$^{\#\alpha}$	1.1 ± 0.1*
Drotaverine (80 mg/kg)	26 ± 0.02*	125 ± 0.06*$^{\alpha}$	41 ± 0.4*	0.38 ± 1.4$^{\#,\alpha}$	3.4 ± 0.06*	656 ± 2*$^{\#\alpha}$	0.8 ± 0.03*$^{\alpha}$

Data are presented as mean ± SEM, n = 10

*P < 0.05 was given when compared with streptozotocin treated group

$^{\#}$P < 0.05 vs control

$^{\alpha}$P < 0.05 vs positive control

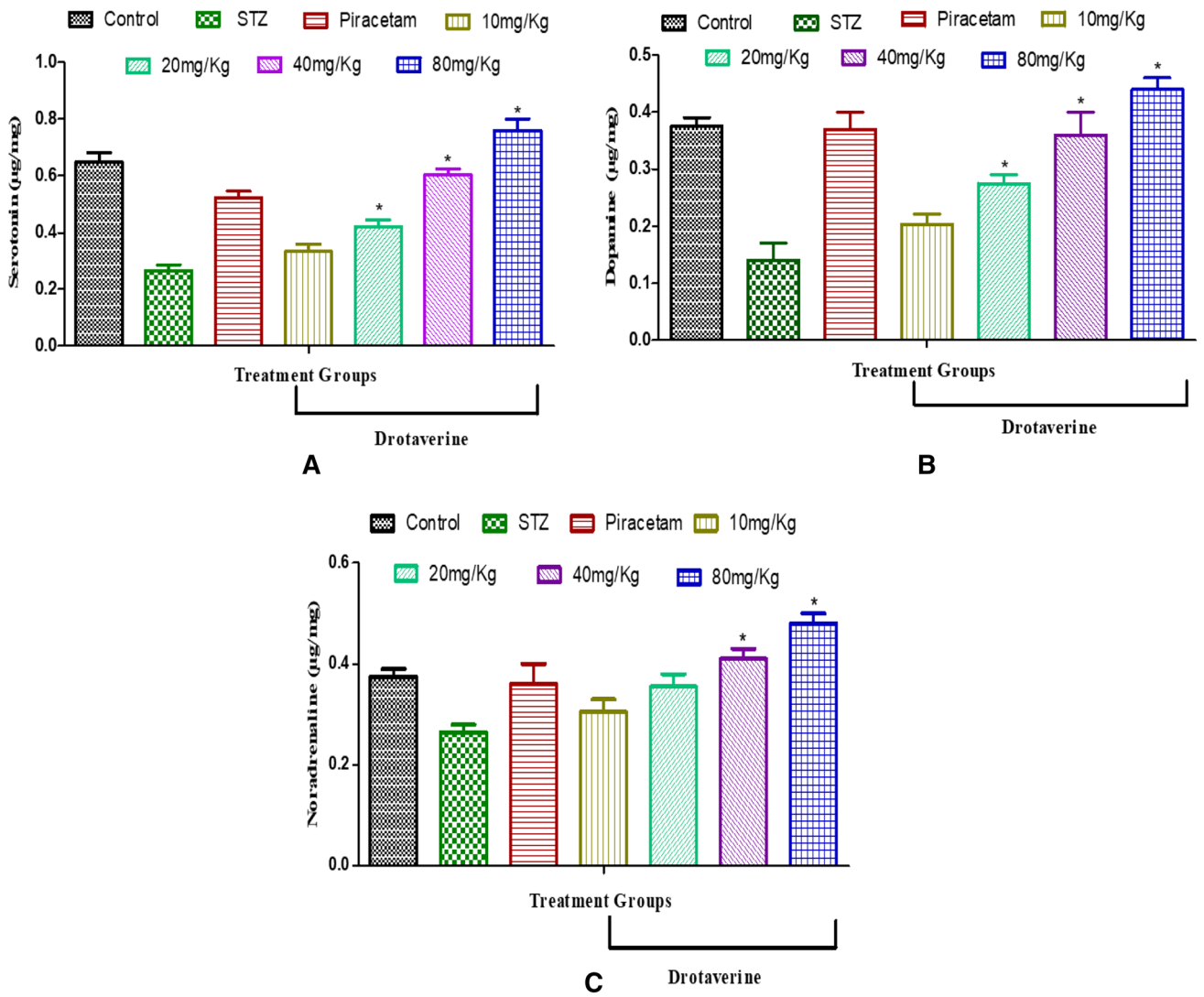


Fig. 6 Effect of drotaverine on neurotransmitters levels in mice brain. Data are presented as mean ± SEM, n = 10. *P < 0.05 as compared with streptozotocin treated group

Effect of Drotaverine on Neurotransmitters in Mice Brain

Drotaverine at dose levels of 20, 40 and 80 mg/kg showed significant (P < 0.05) rise in serotonin, dopamine and noradrenaline levels as compared to STZ group (Fig. 6a–c). Rise in these neurotransmitter levels by drotaverine is believed to be associated with increased spatial learning, and improvement of short term and long term memories.

In-Silico Modeling

Drotaverine was further investigated for its anticholinesterase activity by induced fit docking. Cognate re-docking validated the docking protocol that produced the similar conformation of native ligand with 0.22 root mean square deviation (RMSD) to co-crystallized ligands confirmation (Fig. 7a). The ligands were found to share the same binding pocket as that of co-crystallized ligand (Fig. 7b). The binding score (S) of piracetam serve as standard with − 5.59 kcal/mol conformational energy that depicted its binding affinity towards the active site of AChE. However, the induced fit docking of drotaverine revealed its superior binding affinity, at the active of AChE, with − 9.499 conformational energy as compare to Piracetam (Table 6). The analysis of spatial configuration showed that drotaverine orients itself into a conformation with more active interactions, as compare to Piracetam, with vital residues of AChE active site (Fig. 8). Drotaverine was also found to interact with majority of conserved residues of piracetam's interaction. Comparatively, the structural functionality and conformation of drotaverine allowed it to possess the superior interactions at the binding

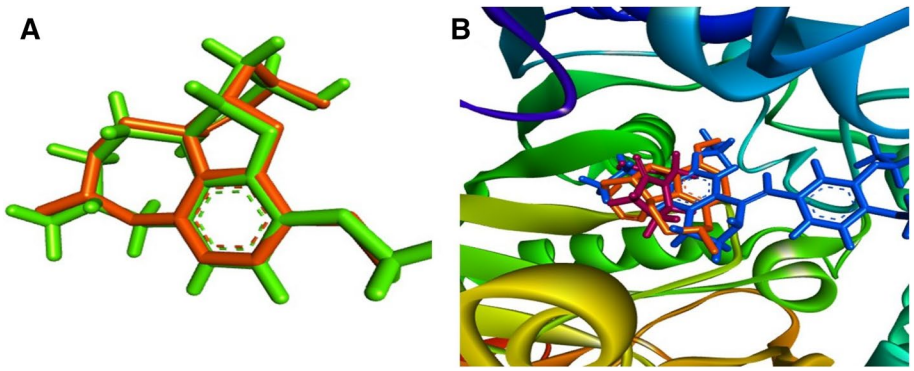


Fig. 7 Induced fit docking of ligands at the AChE active site; **a** The cognate re-docking of native ligand (green) highlighted its same conformation as compare to co-crystallized ligand (orange). **b** piracetam (purple) and drotaverine (blue) shares the same binding pocket of co-crystallized ligand (orange)

Table 6 Binding score, interacting residues and interaction type of piracetam and drotaverine on ACHE

Compound	Binding score (S) kcal/mol	Interacting residues	Interaction type
Piracetam	− 5.59	TYR337, TRP86, HIS447, GLY121, GLY122, SER203, GLU202	H-bonding, π-alkyl
Drotaverine	− 9.499	TYR341, TYR337, TYR124, GLU202, TRP86, SER203, PHE295, TRP236, PHE297, TRP286, SER293	H-bonding, π–π T-shaped, π–π stacked, π-alkyl

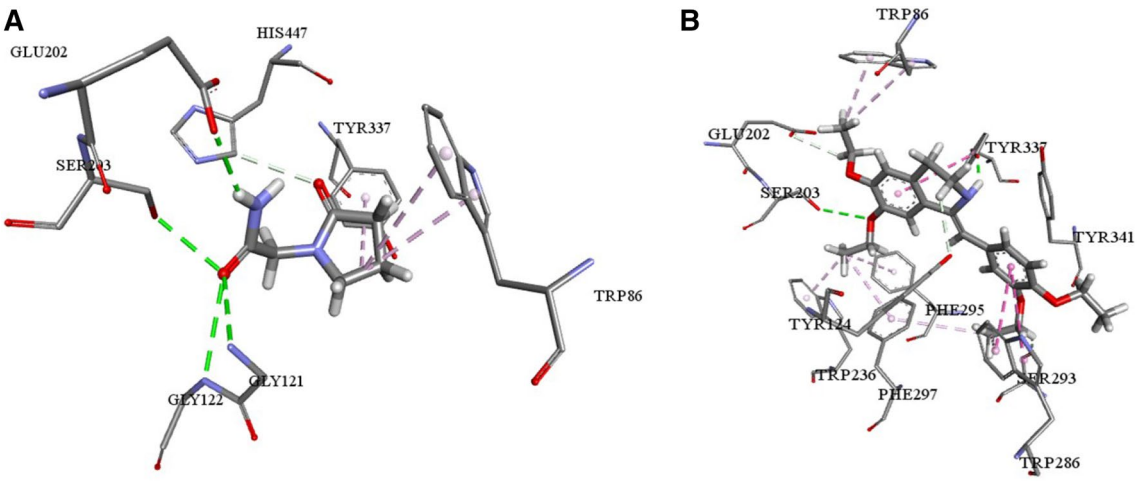


Fig. 8 Conformational analysis of piracetam (**a**) and drotaverine (**b**) at the active site of AChE. Interacting pattern of simulated best binding mode of piracetam (**a**) and drotaverine (**b**) in three dimensional (3D) space

site. Piracetam complex was stabilized by the H-bonding with GLU202, SER203, GLY121 and HIS447 along with π-alkyl bonding with TYR337 and TRP86 residues of AChE active site (Fig. 9). Whereas, drotaverine complex was stabilized with more diverse interactive and distinct pattern of bonding that revealed significant hydrophobic interactions in addition to H-bonding. The drotaverine conformation was stabilized by the π–π stacked and T shaped interactions with TRP286, π-alkyl bonding with TRP86, PHE295, TRP236, PHE297. In addition, the H-bonding with TYR337, TYR341, TYR124, SER203, SER293 and GLU202 also significantly contributed into endurance of drotaverine complexation at the active site of AChE. However, the drotaverine was found to interact with conserved residues, TYR337, TRP86, SER203 and GLU202, of piracetam interactions but with distinct nature of bonding.

Protein Analysis by ELISAs

Selected proteins (beta amyloid and tau) estimation through the Elisa kits indicated that drotaverine at 20, 40 and 80 mg/kg dose levels significantly ($P < 0.001$) reduced the levels

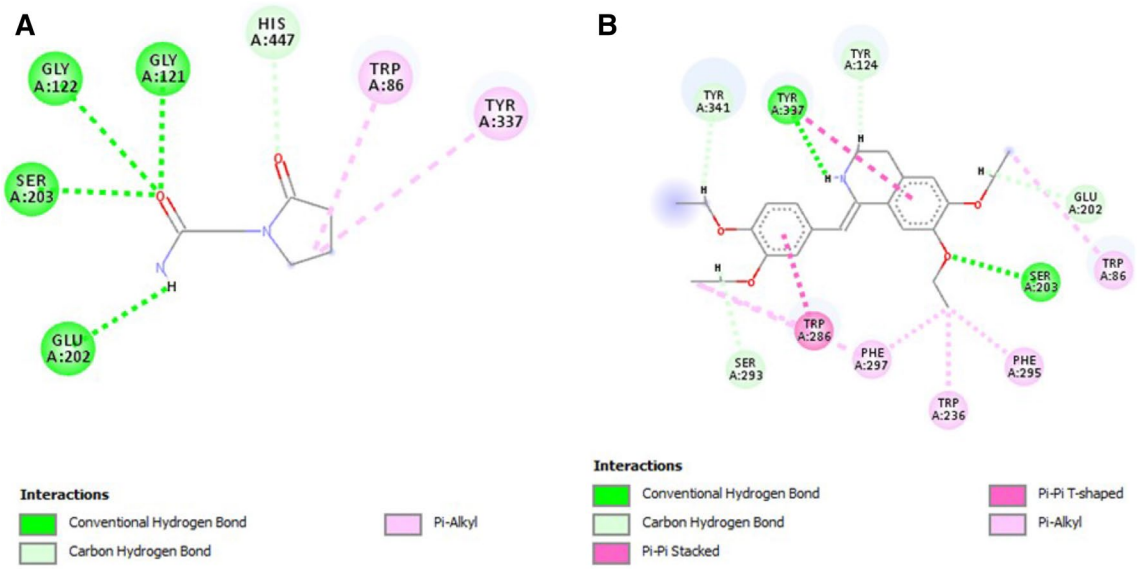


Fig. 9 Piracetam (**a**) and drotaverine (**b**) interactions with residues of AChE active site; piracetam (**a**) and drotaverine (**b**) simulated in two dimensional (2D) perspective to visualize their potential interacting residues illustrated as balls colored by type of interactions

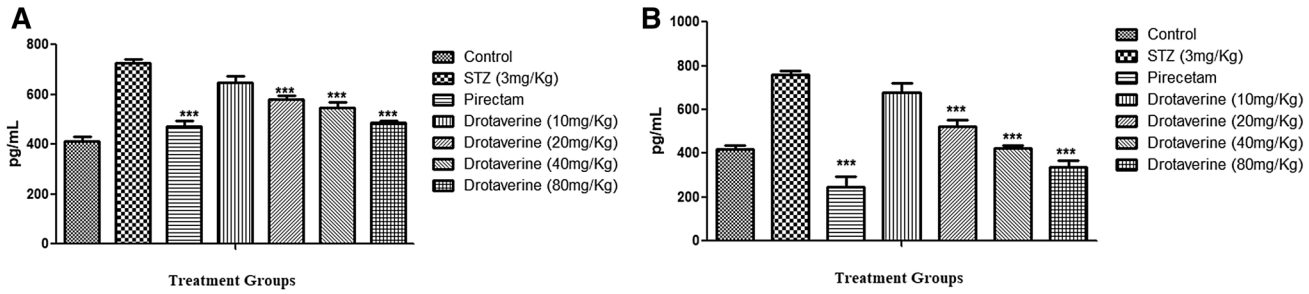


Fig. 10 Estimation of beta amyloid (**a**) and tau (**b**) protein levels in the brain of mice. ***P < 0.001 was given in comparison to STZ treated group

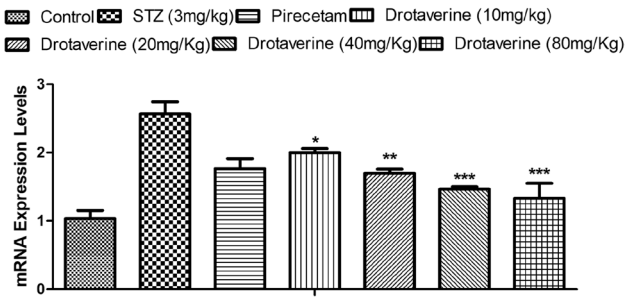


Fig. 11 Effect of drotaverine on mRNA expression analysis of AchE. *P < 0.05, **P < 0.001 & ***P < 0.001 was given in comparison to STZ group

of theses protein in the brain of experimental animals. This reduction of protein levels improves the cognitive behavior in mice (Fig. 10).

AchE Analysis by RT-PCR

The mRNA expression level of acetylcholinesterase (AchE) was decreased upto 1.33 ± 0.21 fold at 40 mg/kg dose when compared with disease (STZ) group (2.56 ± 0.17). Drotaverine decreases the expression levels of mRNA in a dose dependent manner. As the dose increases from 10 to 40 the expression level of AchE were decreased from 2.00 ± 0.058 to 1.33 ± 0.21 (Fig. 11).

Discussion

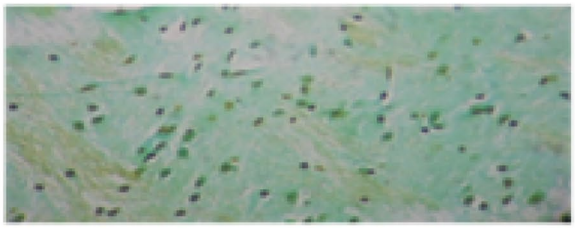
Alzheimer's disease (AD), a disorder associated with loss of synapses and neurons in the brain [7]. become most usual cause of dementia in elderly people due to possible ideology of obesity, inflammation, cardiovascular disorder,

hypercholesterolemia, viral infections and diabetes. Sub-diabetogenic dose of the STZ through ICV route of administration impaired the metabolic energy and oxidative damage states in the brain that leads the cognitive dysfunctioning [35]. This decrease in ATP production resulted the cholinergic deficit in the brain and gives the relevant model for the sporadic Alzheimer disease [36]. The decrease in ATP level resulted the ROS generation, release of inflammatory mediators and accumulation of amyloid beta peptides as well as neurofibrillary tangles of aggregated hyper-phosphorylated tau protein in brain [37]. Due to enormous widespread of AD, it is substantially important to scrutinize the effect of new molecule in memory improvement.

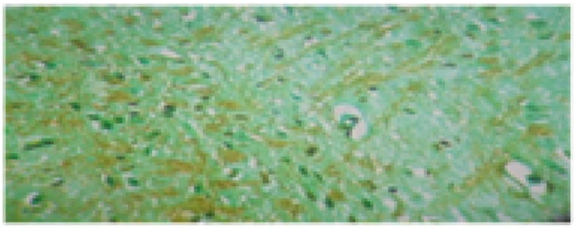
Open field test was employed to assess the locomotors activity, exploration and anxiolytic behavior of animals [38]. Results obtained from open field test manifested that drotaverine at highest dose level of 80 mg/kg highly improved exploration and anxiolytic behavior and as well as improved locomotor activity when compared with STZ-group. Anxiety has been shown to have a link with decline in cognitive reserve. Previous studies revealed that there is a correlation between anxiety and decrease in cognitive performance [39]. According to some studies, anxiety is a out-turn of oxidative damage and inflammation of CNS. Recent studies revealed that there is a bi-directional link between amyloid beta plaques and anxiety. Higher anxiety in individuals with amyloid beta (Aβ) plaques lead to rapid decline in cognitive performance [40]. More time spent in central area showed less anxiety [41]. Elevated plus maze test provided the index of higher level of anxiety [42]. Decrease in the number of entries and reduction of time spent in an open arm is an indicator of anxiety [41]. Drotaverine at dose level 80 mg/kg showed highly significant ($P < 0.001$) decline in transfer latency as well as significally ($P < 0.05$) increased in number of open arm entries and time spent in open arms when compared to STZ-group. Morris water maze test is used for evaluation of spatial learning [24]. It is most accepted model for estimation of learning and memory [43]. On probe test day, drotaverine at dose level of 80 mg/kg significally ($P < 0.05$) showed large number of crossings and more time spent in the target quadrant (north) as well as escape latency was higly decreased ($P < 0.001$) when compared to 1st day and diseased group. The step-down passive avoidance test was used for estimation of long term memory based on the type of learning to inhibit the bahavior of stepdown to escape from punishement [2]. Drotaverine at dose of 80 mg/kg showed increase in retention latency which was highly significant ($P < 0.001$) in contrast with 1st day and STZ-treated group. Drotaverine at dose of 80 mg/kg significantly increased the glutathione, catalase, protein and superoxide dimutase levels as well as decreased the nitrite level which are indicative of decline in oxidative stress and inturn improvement of memory [10]. Drotaverine at all doses showed increase in the malondialdehyde level which is an indicator of lipid peroxidation. Acetylcholinesterase levels were significantly ($P < 0.05$) decreased dose dependently with drotaverine and decline in AchE levels might be due to the indirect acting cholinergic effect of drotaverine; increasing acetylcholine levels by inhibition of actylcholinesterase. Effect of drotaverine in memory improvement may be due to the binding of acetylcholine to the nicotinic receptors (nAChRs) in brain which results in elevation of cytoplasmic calcuim level and stimulate the calcuim dependant intracellular processes such as neurotransmitter release and gene expression which is associated with increase learning and memory improvement [44]. mRNA expression analysis also support the hypothesis of our study as its expression was decreased by PCR studies (Fig. 10). The *in-silico* modelling further delineated the mechanism of drotaverine to inhibit the AChE activity. The molecular docking studies provide the insights into modulatory behavior of drugs by simulating the ligand–receptor complex conformations and interactions between them. The induced fit docking is a unique simulation that copes the structural rearrangement of flexible side chains, of residues at the active site of receptors, upon ligand binding and accurately placing the ligand into the binding site to avoid the false positive results due to receptor flexibility [45]. Drotaverine was found to inhibit the AChE with higher binding affinity as it significantly exceeded the standard's binding score that further supported the experimental inhibition of AChE. This superior binding affinity of drotaverine can be justified by its both hydrophobic and hydrophilic interactions with diversity of vital residues responsible for AChE activity. The diversity of drotaverine interacting residues also included the majority of residues conserved for Piracetam anticholinesterase activity but with distinct nature of bonding. Therefore, the structural functionality, diverse interactions and distinct bonding pattern may substantiate the drotaverine superior binding affinity towards AChE and reasonably supports its experimental inhibition of AChE relative to Piracetam.

According to a study by ding et al., neurotransmitters such as serotonin and nor-adrenaline levels were reduced after intracerebroventricular injection of STZ. The reduction in neurotransmitter titre may be due to decrease in cerebral glucose level and energy metabolism [46]. Another study has shown that 5-HT, noradrenaline and serotonin levels decreased in Alzheimer's disease [47]. Noradrenaline worked by activation of cAMP/protein kinase A [48]. Drotaverine at dose level of 80 mg/kg showed significant ($P < 0.05$) increase in serotonin, dopamine and nor-adrenaline levels when compared with STZ-group. As drotaverine is a selective Phosphodiesterases isoenzyme IV inhibitor [13], it works by elevating the level of cAMP, adenosine triphosphate (ATP) in the presence of adenyl cyclase enzyme resulting in the formation of cAMP. cAMP activates protein

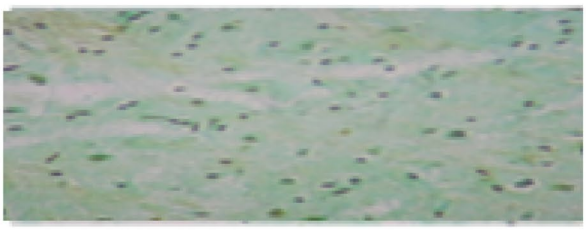
kinase A and results in phosphorylation of cAMP response element binding protein (CREB). CREB is involved in improvement of memory by increasing synaptic plasticity [11]. According to histopathological studies, drotaverine at lower doses showed minor effect against neurodegenration, while at higher doses 40 and 80 mg/kg displayed protective effects with enormous unharmed cells and less neurodegeneration (Fig. 12). Pirecetam was used as a standard in this study. Its acts as a cognitive enhancer due to the improvement in the neurotransmission of acetylcholine. The



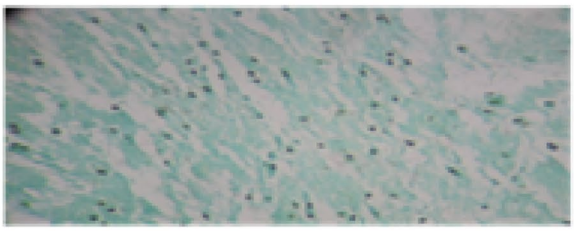
Control group showed normal morphology of brain with intact neurons and other supporting cells



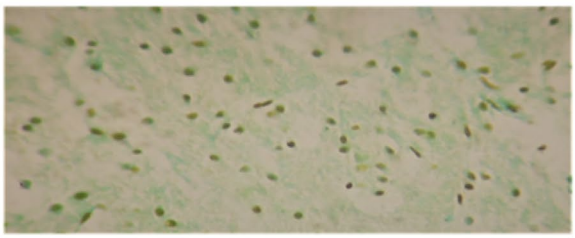
Streptozotocin group showed neurofibrillary tangles, neuritic plaques and damaged cells



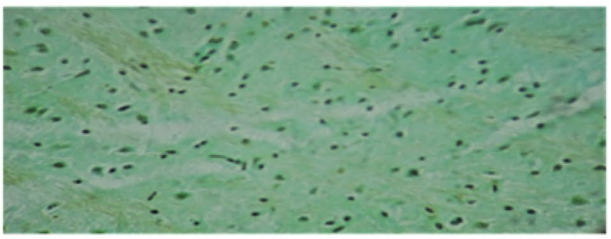
Drotaverine at dose of 10 mg/Kg showed few intact cells with evidence of some spotty degeneration



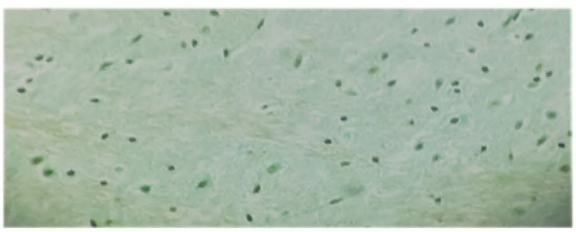
Drotaverine at dos of 20 mg/Kg showed less disruption of neurons and supporting cells



Drotaverine at dose of 40 mg/Kg showed many intact cells and blood vessels and very small area of degeneration changes



Drotaverine at dose of 80 mg/kg showed intact cells and blood vessels as well as glial cells



Piracetam showed many intact cells with other supporting cells

Fig. 12 Histopathological studies of control, piracetam (standard), streptozotocin and treatment groups (drotaverine). Representative of 10 animals in each group

limitation of this study is the use of single model for AD and the use of drotaverine in parallel to STZ.. Further studies should be done on AD by using different models like $AlCl_3$ induced AD model etc.

Conclusion

It can be concluded from the data obtained from this study that drotaverine improved learning and memory.Drotaverine showed increase in long term potentiation, improvement of short term memory and retrieval process. PDE4 inhibitor (Drotaverine) increases the neurotransmitter levels and endogenous antioxidant enzyme levels.The effects of drotaverine were found dose dependently, the dose level of 80 mg/kg exhibiting maximal memory improvement effect.

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Author Contributions SN & FA did the research work and drafted the manuscript. BA & FA supervised the project. ZR & US did the computational activity. MS & AR review the manuscript and performed ELISA analysis. TI analyzed the data and reviewed the manuscript in detail.

Data Availability All Data were generated in-house and that no paper mill was used. Immunoblot technique was not used in this manuscript and all data are included in the original manuscript. There is a no data as supplementary files.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical Approval All methods were approved by research ethical committee of Riphah International University, Lahore with an authorized number of REC/RIPS-LHR/011 ruled under the regulation of National Institute of Health Guide for the Care and Use of Laboratory Animals (1996).

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