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Annals of Biological Research, 2021, 13 (5):159-174
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ISSN 0976-1233
CODEN (USA): ABRNBW

Dulaglutide Improves Aluminum Chloride Induced Cognitive Dysfunction in Diabetes Associated Alzheimer's Rat Model

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Received: 08-Jun-2022, Manuscript No. ABR-22-54319; **Editor assigned:** 10-Jun-2022, PreQC No. ABR-22-54319;

Reviewed: 22-Jun -2022, QC No. ABR-22-54319; **Revised:** 29-Jun-2022, Manuscript No. ABR-22-54319; **Published:** 08-Jul-2022 DOI:10.4172/0976-1233.004

ABSTRACT

Background: Type-2 diabetes is an etiological factor for Alzheimer's. GLP-1 analogues were prescribed in treating type-2 diabetes mellitus, where the presence of GLP-1 receptors in brain also makes it a potential target for Alzheimer's disease. **Aim:** The aim of the study is to evaluate the neuroprotective effect of dulaglutide in aluminum chloride induced cognitive dysfunction in type-2 diabetes associated Alzheimer's experimental rats by conducting behavioral studies, estimating the biochemical parameters and histopathological changes involved in the pathogenesis of the disease.

Objective: To evaluate the effect of GLP-1 analogue on learning and memory in Aluminum chloride induced Alzheimer's disease in type-2 diabetic rats by using physical and biochemical methods.

Methods: Study design included male wistar rats made into 6 groups (n=9) where group 1 was treated as vehicle control, group 2 as diabetic control, group 3 as Alzheimer's control, group 4 as diabetic and Alzheimer's control, group 5 as standard that received donepezil 3 mg/BSA p.o, group 6 as test that received dulaglutide 1.5 mg/BSA. Type-2 diabetes was induced by administrating Nicotinamide (NA) 120 mg/kg i.p followed by streptozotocin (STZ) 30 mg/kg i.v. Alzheimer's was induced by administrating aluminum chloride 100 mg/kg oral after 3 days of diabetes induction. This study was conducted for a period of 42 days. Behavioral assessments were made on day 0, 21 and 42 using elevated plus maze, Y-maze, Actophotometer and Novel Object Recognition test (NOR) followed by the estimation of biochemical parameters such as blood glucose, protein content, Brain specific Creatine kinase (CK-BB), Acetylcholine (ACh), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO), Superoxide Dismutase (SOD), Reduced Glutathione (GSH) and Catalase (CAT) activity.

Result: Manifested that Dulaglutide (1.5 mg/BSA) improved cognitive function in rats providing a significant reduction in blood glucose levels (114.65 ± 8.87 mg/dL), protein content (8.907 ± 7.18 mg/g), CK-BB (219.57 ± 9.23 U/L), Acetylcholinesterase (AChE) activity (0.593 ± 5.83 μ M/mg) and lipid peroxidation (2.78 ± 7.69 μ M/mg) and also presented a significant improvement in Acetylcholine (ACh) levels (7.321 ± 7.52 μ M/mg), SOD (51.14 ± 10.42 U/mg), GSH (11.09 ± 10.68 μ M/mg) and catalase activity (1.12 ± 11.75 μ M/mg) when compared with diseased controls which were analyzed using one-way ANOVA followed by Post-Hoc Tukey's test with a significant variance of $P < 0.05$. Histopathological study of hippocampus with Congo red and hematoxylin and eosin (H and E) stain revealed a decrease in the plaque deposition and degenerated neurons respectively.

Conclusion: These findings from the study manifest the neuroprotective effect of dulaglutide against aluminum chloride induced cognitive dysfunction in Type-2 diabetic Alzheimer's rats.

Keywords: Alzheimer's disease, Aluminum Chloride, Dulaglutide, GLP-1 analogues, Neuroprotection, Nicotinamide, Streptozotocin, Type-2 Diabetes Mellitus

Abbreviations: GLP-1: Glucagon-like Peptide-1; NFTs: Neuro Fibrillary Tangles; NMDA: N-Methyl-D-Aspartate Receptor; GIP: Glucose-dependent Insulinotropic Polypeptide; PKB/Akt: Protein Kinase B; PI3K: Phosphoinositide 3-Kinase; MAPK: Mitogen Activated Protein Kinase; APP: Amyloid Precursor Protein

INTRODUCTION

Alzheimer's Disease (AD), as described by Alois Alzheimer is a progressive neurodegenerative disorder characterized by memory loss, neuronal degeneration, deposition of β -amyloid plaques and Neurofibrillary Tangles (NFTs) [1,2]. Risk factors for Alzheimer's disease include genetic, physiological, environmental factors [3-7]. Pathophysiology involves hypothesis related to amyloid, cholinergic, glutamatergic and oxidative stress ultimately causing neuronal and memory loss [8-10]. There is no specific diagnostic test available for the detection of AD, but physical methods for cognitive assessment and brain imaging tools for A β deposition were found to be useful [11-13]. Treatment includes cholinesterase (acetylcholinesterase) inhibitors such as donepezil, galantamine, rivastigmine, tacrine [14,15] and NMDA antagonist memantine [16]. Type-2 diabetes and AD share common potential mechanisms by which it was found that type-2 diabetes is an etiological factor for the development AD in diabetic patients and this condition is often termed as 'Type-3 diabetes' [17,18]. AD progresses in Type-2 diabetes as a result of impaired glucose and energy metabolism, insulin resistance, amyloidogenesis, elevated Advanced Glycation End Products (AGEPs) and oxidative stress [19-21]. Incretins (Intestinal secretion of Insulin) namely GLP, GIP are known as gut hormones secreted from enteroendocrine cells exerting their major effect on glucose homeostasis [22-25]. GLP-1 and its analogues exerts insulinotropic effect by acting on GLP-1 receptors present on the beta cells of pancreas thereby stimulating insulin release and reducing the glucagon secretion [26-30]. GLP-1 analogues have found to have a neuroprotective role in Type-2 diabetic associated AD as GLP-1 receptors occur in different parts of the brain primarily in the area that is necessary for memory and cognitive functions i.e., cortex and hippocampus [31-35]. Dulaglutide, a once weekly GLP-1 analogue was found to have the memory enhancing and neuroprotective activity in induced animal models [36-39]. They bind with the GLP-1 receptor present on the cell membrane thereby activating two signaling pathways PI3K-PKB/Akt pathway and MAPK [40-42]. Activation of receptors stimulates PI3K with conversion of PIP2 to PIP3. PKB/Akt is activated by binding with PIP3 mediated by PDK. Activation of Akt/PKB causes inhibition of apoptosis promoting neuronal cell survival. Receptor activation also activates the cAMP pathway stimulating the protein kinase-A which is involved in gene transcription promoting neuronal and synaptic growth and potentiates regeneration of neurons [43-45].

Aluminum is a non-essential abundant non-redox metal ion existing in the environment but prolonged exposure to aluminum makes it a neurotoxin affecting major areas of brain [46-47]. Aluminum chloride was found to have a role in potentiating the formation of A β and NFTs in brain which makes it a source of AD inducer [48]. Aluminum can easily cross BBB and produces various toxic effects in the brain. Aluminum induced neurodegeneration is associated with conditions such as overexpression of APP, potentiating the accumulation of A β , aggregation of tau proteins and apoptosis [49,50]. It interferes with the microtubule filaments causing an integrational change in its structure and interferes with the phosphorylation of tau which causes it to accumulate in the form of NFTs [51]. It also increases oxidative damage and activates the enzyme caspase which promotes neuronal cell death [52].

MATERIALS AND METHODS

Drugs and chemicals

Dulaglutide subcutaneous injection (TRULICITY 1.5 mg/0.5 ml by Eli Lilly) and donepezil tablets were purchased from a local pharmacy store. Streptozotocin was purchased from SRL laboratories, India. Acetylcholine iodide was purchased from SRL laboratories, India. Nicotinamide, aluminum chloride and other chemicals required for the study were obtained from a local chemical vendor. Assay kits required for the estimation of biochemical parameters were obtained from ERBA Mannheim limited.

Equipment

Digital weighing balance was used to measure the chemicals. Cold centrifugation system (REMI CM-12) was used to separate serum from collected animal blood samples. Semi auto analyzer was employed to analyze the biochemical constituents in serum samples. Tissue homogenizer was used to homogenize the isolated animal's brain. UV-visible spectrophotometer was used to determine the biomarkers from tissue homogenate.

Experimental animals

Healthy adult male albino rats (wistar strain) of age 13-15 weeks old weighing between 200-250 grams were obtained from a licensed animal vendor. They were housed in cages (3-4 rats/cage) and acclimatized for 1 week in standard laboratory conditions with temperature maintenance of $25 \pm 2^\circ\text{C}$. They were fed with standard pellet diet and had free access to food and water. The experimental protocol was approved by Institutional Animal Ethics committee (CPC-SEA/1657/IAEC/CMRCP/COL-19/75) for the experimentation and care on animals.

Allotment of experimental animals

After the acclimatization period, animals were assessed for health condition and weight. Healthy and animals with an average weight of 200-250 grams were selected and grouped into six with nine animals in each group. Group 1 was considered as control. Group 2 was considered as diabetic control. Group 3 was considered as Alzheimer's disease control. Group 4 was considered as diabetic and Alzheimer's disease control. Group 5 received standard treatment induced with diabetes and Alzheimer's disease. Group 6 received the test drug treatment.

Induction of disease into experimental animals

Induction of type-2 diabetes: Animals were fasted overnight. Nicotinamide 120 mg/kg was given through intraperitoneal route followed by intravenous administration of streptozotocin 30 mg/kg after 30 minutes of nicotinamide administration. Elevated blood glucose levels (150-200 mg/dL) were observed after 3 days indicating the development of Type-2 diabetes as a result of beta cell destruction in pancreas [53-54]. Care was taken by keeping them under controlled feeding conditions.

Induction of alzheimer's disease: Alzheimer's disease was induced into Type-2 diabetes rats by the daily oral administration of aluminum chloride 100 mg/kg for an experimental time period of forty two days [55].

Drug treatment

- i) Donepezil 3 mg was calculated based on the body surface area and it was administered as a suspension (0.5 ml) through oral route daily in the evening throughout the experimental period [56].
- ii) Dulaglutide was administered through subcutaneous route at a weekly once interval throughout the experimental period [57].

Assessment of memory and cognition in experimental animals

Behavioral performance was assessed on 0, 21st and 42nd day by employing different models such as elevated plus maze, Y-maze, actophotometer and Novel Object Recognition (NOR) test.

Elevated plus maze: Elevated plus maze was considered as a model for assessing long-term memory in experimental animals. This maze was elevated at a height of 50 cm with two closed arms measuring 50 cm \times 10 cm \times 40 cm and two open arms measuring 50 cm \times 10 cm emerging from the center of the maze in four directions. During the experiment, each animal was placed individually at one of the open arm facing away from the center and the time taken for the animal to reach the closed arm was recorded as Transfer Latency (TL). Memory acquisition was recorded only when the animal was able to reach the closed arm within the assigned time (3 minutes) [58,59].

Y-maze: Spontaneous/forced alteration is a result of hippocampal damage causing memory deficits which was assessed by using Y-maze. It comprised of three equal arms (A, B, C) arranged at an angle of 120° measuring 50 cm \times 10 cm \times 15 cm. During the training period each animal was positioned at arm A facing opposite to the center and were trained to locate the food placed in one of the arms (B) by blocking the other (C) within an allotted time (5 minutes). During the testing period, the same procedure was conducted without blocking the third arm. Number of entries into the novel arm (C) was recorded as Forced alterations and the percent time spent in the novel arm was recorded [60,61].

Actophotometer: Actophotometer was used to study the ambulatory movements of experimental animals that can be ascribed to cognition dysfunction. Actophotometer consists of a closed chamber with a removable lid at the top. It is provided with an electric grid floor and photocell that detects the light beam obstruction caused by the animal, it reflects the locomotor movement which can be read on the digital display. Each animal was placed individually inside the actophotometer and the lid was closed before operating and recorded for 10 minutes. Locomotor index was calculated on different days (0, 21 and 42) that give a measure of locomotor alterations in different experimental groups [62,63].

Novel Objects Recognition (NOR) test: Novel object recognition was used to assess the memory deficits in experimental rats. This test was performed in an open field where the animals were familiarized with the field before conducting the experiment. In the training phase, two similar objects were placed in the open field and the animals were freely allowed to explore the objects. During the experiment, one of the objects has been replaced with an unfamiliar object but has the similar size. Now the animal was allowed to explore with the objects for five minutes. The time spent by the animal with each object was recorded which gives a measure of discrimination index [64,65].

Estimation of biochemical parameters

Estimation of glucose levels in serum: Measurement of serum glucose levels was done by GOD-POD method. This method is based oxidation of glucose molecules present in the sample by the enzyme Glucose Oxidase (GOD) forming a salt of gluconic acid i.e. gluconate and hydrogen peroxide (H_2O_2). The latter undergoes further oxidation with phenol and 4-Aminoantipyrine (4-AAP) catalyzed by Peroxidase (POD) producing quinoneimine red colored dye, whose color intensity is proportional to the glucose concentration in the sample measured at 505 nm [66].

Estimation of Acetylcholine (ACh): Acetylcholine levels was measured by the method proposed by Hestrin, which involves reaction between acetyl group and hydroxylamine forming acetohydroxamic acid and a reddish brown colored complex with ferric ions in acidic medium. Intensity of the color produced is proportionate with the acetylcholine content measured at 540 nm and was given by μM of ACh/mg of tissue [67].

Estimation of Acetylcholinesterase Activity (AChE): Acetylcholinesterase (AChE) is an essential enzyme responsible for the degradation of acetylcholine which is found to be increased in diseased conditions like Alzheimer's. Ellman method was employed to measure acetylcholinesterase activity. This is given by μM of AChE/mg of tissue [68].

Estimation of total protein content: Total protein content estimation was carried out by Biuret method involving a biuret reaction between the peptide bonds of the protein present in the sample and copper II ions in alkaline medium forming a blue-violet colored ion complex measured at 546 nm. It was given by mg of protein/g of tissue [69].

Creatine Kinase Isoenzyme (CK-BB): CK-BB is an isoenzyme of creatinine kinase that is localized predominantly in brain. This method is based on the coupling reaction between creatine phosphate and Adenosine Diphosphate (ADP) mediated by creatine kinase enzyme resulting in creatine and Adenosine Triphosphate (ATP). ATP further phosphorylates glucose molecules to glucose-6 phosphate catalyzed by hexokinase. Glucose-6 phosphate is oxidized with NADP in the presence of glucose-6 phosphate dehydrogenase yielding gluconate-6 phosphate and NADPH. The absorbance of latter was measured at 340 nm which is proportional to the creatine kinase activity present in the sample [70].

Estimation of Lipid Peroxidation (LPO): Lipid peroxidation was used as a measure to estimate the free radical generation as a result oxidation of lipids in living cells. This method is based on the principle reaction between MDA and TBA producing pink color solution which was then measured spectrophotometrically at 535 nm. The result was expressed as μM of MDA/mg of protein [71].

Estimation of Superoxide Dismutase (SOD): Superoxide dismutase is an antioxidant enzyme present in living cells to defend the superoxide radicals. The principle involved in this method is to estimate the activity of SOD based on the inhibition of autoxidation of pyrogallol which is measured from absorbance at 420 nm. The activity was expressed as units/minute/mg protein [72].

Estimation of Reduced Glutathione (GSH): Reduced glutathione is the active form of glutathione that acts by scavenging the free radicals generated in the body from cellular pathways. The principle involved in Ellman's method was

based on the reaction between GSH and Ellman's reagent to form a yellow colored product whose absorbance was measured spectrophotometrically at 412 nm. The result was expressed as μM of GSH/mg of protein [73].

Estimation of catalase: Catalase is an essential antioxidant required for the catalytic reduction of hydrogen peroxide into water and oxygen. The principle involved in this assay is based on the reduction of hydrogen peroxide by catalase followed by the termination of reaction using ammonium molybdate. This unreacted hydrogen peroxide forms a yellow colored complex with ammonium molybdate and the color intensity was determined at 374 nm by a spectrophotometer. The enzymatic activity was given as U/mg protein [74].

Histopathological examination: Isolated rats brain was fixed in 10% buffered formalin solution. This step is followed by tissue processing which includes dehydration using alcohol. Dehydrated tissues were embedded in paraffin wax ($56\text{ }^{\circ}\text{C}$ to $62\text{ }^{\circ}\text{C}$) for 24 hours and then made into thin sections ($8\text{ }\mu\text{m}$) with a microtome. The processed tissues were mounted on a microscopic slide and deparaffinised for staining with congo red and Hematoxylin and Eosin (H and E) for histopathological study using a light microscope [75,76].

Statistical analysis: Results obtained were represented as Mean \pm SEM and the statistical significance was set at $p < 0.05$. One-way ANOVA was used for the comparative analysis between the groups followed by post-hoc multiple comparisons using Tukey's test in Graph Pad prism software.

RESULTS AND DISCUSSION

Body weights

Results presented in Table 1 indicates Dulaglutide produced a reduction in body weight of the animals from day 0 to day 42 with 224 ± 3.51 when compared to diabetic controls showing an increasing in body weight indicating diseased state with 240 ± 1.53 in diabetic control and 232 ± 3.16 in D+A. Standard and normal groups did not show notable difference. This shows the anti-diabetic potential of dulaglutide in controlling diabetes and obesity. The average body weights of experimental animals on day 0, 21 and 42 are depicted in Figure 1.

Table 1. Effect of dulaglutide on body weight

Day	Vehicle	Diabetic control (D)	Alzheimer's control (A)	D+A	D+A +donepezil (3 mg/BSA)	D+A +dulaglutide (1.5 mg/BSA)
Day 0	215 ± 4.08	224 ± 1.53	235 ± 4.65	219 ± 3.27	223 ± 3.57	243 ± 2.47
Day 21	209 ± 3.96	$230 \pm 2.23^*$	$230 \pm 3.74^*$	$225 \pm 2.14^\#$	228 ± 3.57^a	$238 \pm 3.57^\#$
Day 42	200 ± 3.27	$240 \pm 1.53^*$	$220 \pm 3.51^*$	$232 \pm 3.16^\#$	238 ± 4.01^a	$224 \pm 3.51^\#$

Note: Values were represented as mean \pm SEM (n=9). Statistical analysis of data was performed using One-way ANOVA followed by post-hoc Tuckey's test. * $p < 0.05$ when compared with normal, $^\#$ $p < 0.05$ when compared with diabetic control, a $p < 0.05$ when compared with D+A control.

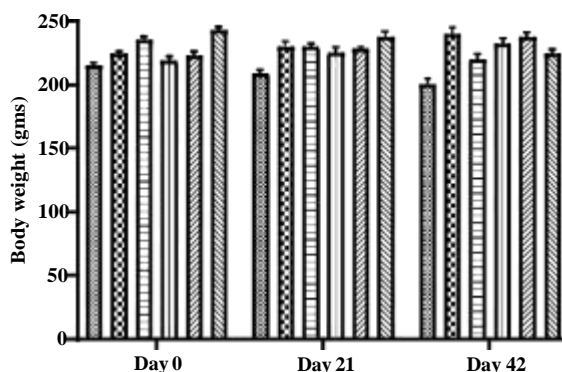


Figure 1. Representation of effect of dulaglutide on body weight. **Note:** () Control, () Diabetic Control, () Alzheimer's control, () Diabetic+ Alzheimer's control, () Donepezil(3 mg/BSA), () Dulaglutide(1.58 mg/BSA)

Elevated plus maze

The transfer latency of diseased animals as shown in Table 2 was found to be increased throughout the experimental period from day 0 to 42 when compared with the vehicle control (20.23 ± 1.88) with (D+A) group having an increased latency of 85.56 ± 1.88 during the last day where diabetic control and Alzheimer's control group had 66.17 and $75.1.69$ respectively. Treatment group presented a decrease in the transfer latency among all the three diseased groups with 25.73 ± 2.08 and the standard group with 28.45 ± 1.83 but standard and test group showed less variation. This indicates the ability of the dulaglutide to improve memory and cognition in diseased animals as a function of neuroprotective activity. Effect of dulaglutide on latency on day 0, 21 and 42 are shown in Figure 2.

Table 2. Effect of dulaglutide on transfer latency on day 0, 21 and 42.

Day	Vehicle	Diabetic control (D)	Day control (A)	D+A	D+A +donepezil (3 mg/BSA)	D+A +dulaglutide(1.5 mg/BSA)
Day 0	54.66 ± 1.49	55.75 ± 1.79	56.01 ± 2.08	54.21 ± 2.46	53.45 ± 2.22	64.27 ± 1.59
Day 21	29.15 ± 1.76	$59.09 \pm 2.06^*$	$62.43 \pm 2.04^*$	70.93 ± 2.31^S	41.82 ± 1.71^{aS}	49.66 ± 2.32^{ac}
Day 42	20.23 ± 1.88	$66.17 \pm 2.45^*$	$75.78 \pm 1.69^*$	85.56 ± 1.88^S	28.45 ± 1.83^{aS}	25.73 ± 2.08^{ac}

Note: Values were represented as mean \pm SEM (n=9). Statistical analysis of data was performed using One-way ANOVA followed by post-hoc Tuckey's test. * p<0.05 when compared with normal, S p<0.05 when compared with Alzheimer's control, aS p<0.05 when compared with D+A control, ac p<0.05 when compared with standard.

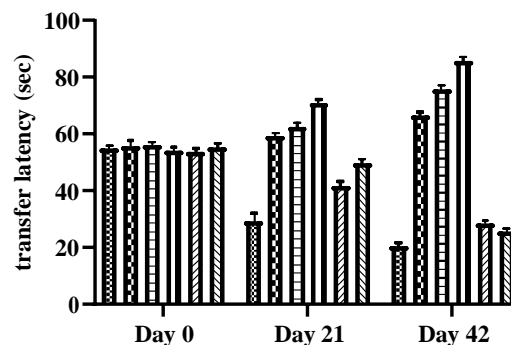


Figure 2. Representation of effect of dulaglutide on transfer latency in experimental animals. **Note:** () Control, () Diabetic Control, () Alzheimer's control, () Diabetic+ Alzheimer's control, () Donepezil(3 mg/BSA), () Dulaglutide(1.58 mg/BSA)

Y-maze: Alteration in memory is a part of diseased condition and the diseased groups shown an increase in the time spent in the novel arm with D+A having the greater alteration with 75.67 ± 3.11 when compared with other diseased controls having 72.55 ± 3.96 in Diabetic control and 71.9 ± 2.04 in Alzheimer's control. Treatment group shown a progressive decrease in the alteration with 46.33 ± 2.37 when compared with diseased groups but significantly less when compared with standard group alteration with 49.66 ± 1.99 . This indicates the effectiveness of the dulaglutide in improving memory deficits in experimental rats (Table 3). Improvement in memory of animals from day 0 to 42 was depicted in Figure 3.

Table 3. Effect of dulaglutide on memory on day 0, 21 and 42

Day	Vehicle	Diabetic control (D)	Day control (A)	D+A	D+A +donepezil (3 mg/BSA)	D+A +dulaglutide(1.5 mg/BSA)
Day 0	67.01 ± 2.69	62.11 ± 2.37	61.34 ± 2.86	65.34 ± 2.06	$63.23 \pm 2.54^*$	$64.45 \pm 2.21^*$
Day 21	59.82 ± 3.45	$67.89 \pm 2.58^*$	$68.04 \pm 2.33^*$	69.11 ± 2.15^S	56.35 ± 1.68^{aS}	57.66 ± 2.45^{ac}
Day 42	49.43 ± 3.78	$72.55 \pm 3.96^*$	$71.9 \pm 2.04^*$	75.67 ± 3.11^S	49.66 ± 1.99^{aS}	46.33 ± 2.37^{ac}

Note: Values were represented as mean \pm SEM (n=9). Statistical analysis of data was performed using One-way ANOVA followed by post-hoc Tuckey's test. * p<0.05 when compared with normal, S p<0.05 when compared with Alzheimer's control, aS p<0.05 when compared with D+A control, ac p<0.05 when compared with standard

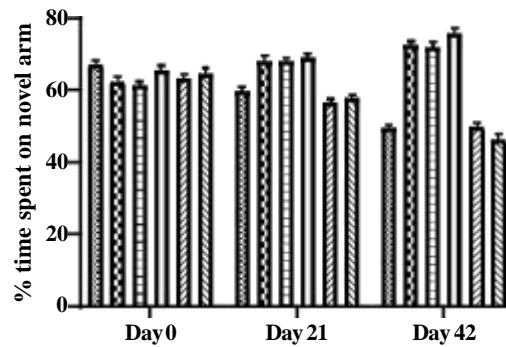


Figure 3. Representation of effect of dulaglutide on memory in experimental animals. **Note:**(▨)Control, (▩) Diabetic Control,(▧) Alzheimer’s control, (▥) Diabetic+ Alzheimer’s control,(▦) Donepezil(3 mg/BSA), (▨)Dulaglutide (1.58 mg/BSA)

Actophotometer: Locomotor activity of diseased controls was found to be decreased with diabetic control 169 ± 1.15 , Alzheimer’s control 149 ± 2.62 and (D+A) group with 140 ± 1.47 when compared to vehicle control activity 230 ± 2.01 . Treatment group had shown a progressive improvement in the locomotor function from day 0 to 42 i.e., 225 ± 2.36 when compared with diseased groups but significantly less when compared with standard group (230 ± 1.66) (Table 4).

Improvement in locomotor activity in experimental animals is an indication of neuroprotective activity of the test drug in treating AD shown in Figure 4.

Table 4. Effect of dulaglutide on novel object recognition on day 0, 21 and 42

Day	Vehicle	Diabetic control (D)	Alzheimer's control (A)	D+A	D+A +donepezil (3 mg/BSA)	D+A +dulaglutide(1.5 mg/BSA)
Day 0	0.42 ± 6.47	0.38 ± 4.45	0.36 ± 6.26	0.4 ± 6.03	0.41 ± 5.48	0.39 ± 5.59
Day 21	0.45 ± 5.26	$0.32 \pm 6.12^*$	$0.28 \pm 5.69^*$	0.23 ± 6.47^s	0.5 ± 5.61^{as}	0.47 ± 5.48^{ac}
Day 42	0.51 ± 5.36	$0.26 \pm 5.67^*$	$0.22 \pm 6.45^*$	0.14 ± 5.31^s	0.55 ± 5.26^{as}	0.56 ± 4.83^{ac}

Note: Values were represented as mean \pm SEM (n=9). Statistical analysis of data was performed using One-way ANOVA followed by post-hoc Tuckey’s test. * p<0.05 when compared with normal, s p<0.05 when compared with Alzheimer’s control, a p<0.05 when compared with D+A control, c p<0.05 when compared with standard

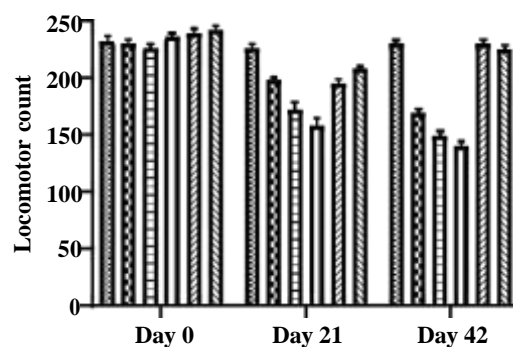


Figure 4. Representation of effect of dulaglutide on locomotor activity in experimental animals. **Note:** (▨) Control,(▩) Diabetic Control, (▧) Alzheimer’s control, (▥) Diabetic+ Alzheimer’s control,(▦) Donepezil(3 mg/BSA), (▨) Dulaglutide(1.58 mg/BSA)

Novel Object Recognition test (NOR): Discrimination Index (DI) shown in Table 5 was calculated from novel object recognition test which provided a decrease in DI in diseased groups D+A, diabetic and Alzheimer's with 0.14 ± 5.31 , 0.26 ± 5.67 and 0.22 ± 6.45 respectively when compared to vehicle control DI of 0.51 ± 5.36 . This was improved in treatment group with 0.56 ± 4.83 but standard group and test groups did not show much significance. Improvement in the DI of experimental animals by the test drug is an indicative of its neuroprotective and memory enhancing activity which was depicted in Figure 5.

Table 5. Effect of dulaglutide on locomotor activity on day 0, 21 and 42.

Day	Vehicle	Diabetic control (D)	Day control (A)	D+A	D+A +donepezil (3 mg/BSA)	D+A +dulaglutide(1.5 mg/BSA)
Day 0	232 ± 2.15	230 ± 0.96	226 ± 0.82	236 ± 1.79	239 ± 1.69	242 ± 1.76
Day 21	226 ± 1.06	198 ± 0.98*	172 ± 2.34*	158 ± 1.36 ^s	195 ± 2.03a ^s	208 ± 1.85 ^{ac}
Day 42	230 ± 2.01	169 ± 1.15*	149 ± 2.62*	140 ± 1.47 ^s	230 ± 1.66 a ^s	225 ± 2.36 ^{ac}

Note: Values were represented as mean ± SEM (n=9). Statistical analysis of data was performed using One-way ANOVA followed by post-hoc Tuckey's test. *,^{*}p<0.05 when compared with normal, ^sp<0.05 when compared with Alzheimer's control, ^ap<0.05 when compared with D+A control, ^cp<0.05 when compared with standard.

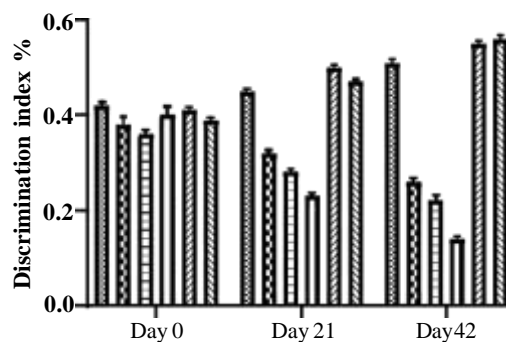


Figure 5. Representation of effect of dulaglutide on novel object recognition in experimental animals. Note: () Control, () Diabetic Control, () Alzheimer's control, () Diabetic+ Alzheimer's control, () Donepezil (3 mg/BSA), () Dulaglutide (1.58 mg /BSA)

Biochemical parameters

Blood glucose levels were estimated in experimental groups and it was found to have immense rise in diabetic diseased groups with 166.625 ± 7.64 in diabetic control and 163.63 ± 5.18 in D+A group. Test drug treatment shown a significant decrease in blood glucose levels after 42 treatment period with 114.65 ± 8.87 while the Alzheimer's standard group remained with 146.25 ± 6.35 (Figure 6). This indicates the anti-diabetic property of test drug showing efficient decrease in blood glucose levels in experimental rats (Table 6). Acetylcholine and acetylcholinesterase are important biomarkers for brain damage. These levels indicated in Table 6 vary in different experimental groups showing decrease in acetylcholine levels and increase in acetylcholinesterase in diseased animals when compared to vehicle control (6.914 ± 4.67 and 0.589 ± 6.75). Acetylcholine levels were found to be 3.709 ± 6.66 in diabetic control, 1.653 ± 6.54 in Alzheimer's control, 0.893 ± 7.32 in D+A group. Standard and test groups showed effective improvement in acetylcholine levels and profound decrease in acetylcholinesterase when compared to diseased control groups but the test group effect was significantly lower than standard drug treatment. ACh and AChE levels in standard group were 7.922 ± 8.61 and 0.511 ± 4.27 respectively, ACh and AChE levels in test group were 7.321 ± 7.52 and 0.593 ± 5.83 (Figure 7). Protein content levels in diseased groups were found to be increased when compared to vehicle group (9.784 ± 4.11). Protein levels of diseased groups were as follows 12.104 ± 6.31 in diabetic control, 17.927 ± 5.29 in Alzheimer's control, 23.456 ± 5.55 in D+A group. Standard and test groups lowered these levels with 9.112 ± 6.07 and 8.907 ± 7.18 respectively (Figure 8). CK-BB levels were lowered with test drug treatment and standard group with 219.57 ± 9.23 and 223.89 ± 7.66 when compared to diabetic control with 621.67 ± 5.42 , Alzheimer's control with 660.61 ± 7.16 , D+A group with 691.32 ± 8.41 (Figure 9).

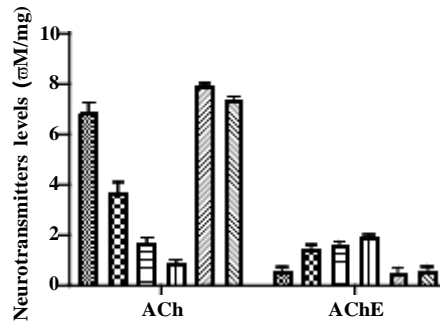


Figure 6. Representation of effect of dulaglutide on blood glucose levels. Note: (Control), (Diabetic Control), (Alzheimer's control), (Diabetic+ Alzheimer's control), (Donepezil(3 mg/BSA)), (Dulaglutide(1.58 mg/BSA))

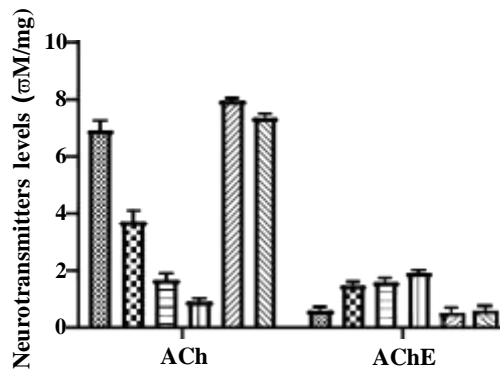


Figure 7. Representation of effect of dulaglutide on Neurotransmitter levels. Note: (Control), (Diabetic Control), (Alzheimer's control), (Diabetic+ Alzheimer's control), (Donepezil (3 mg/BSA)), (Dulaglutide (1.58 mg/BSA))

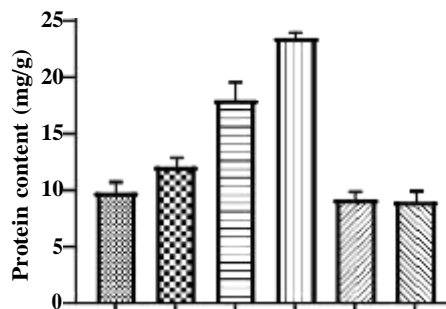


Figure 8. Representation of effect of dulaglutide on protein content. Note: (Control), (Diabetic Control), (Alzheimer's control), (Diabetic+ Alzheimer's control), (Donepezil(3 mg/BSA)), (Dulaglutide(1.58 mg/BSA))

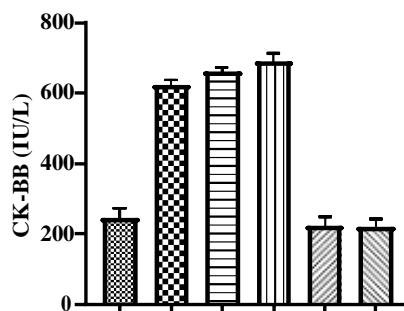


Figure 9. Representation of effect of dulaglutide on CK-BB levels. **Note:** (▨) Control, (▩) Diabetic Control, (▧) Alzheimer's control, (▦) Diabetic+ Alzheimer's control, (▥) Donepezil(3 mg/BSA), (▤) Dulaglutide(1.58 mg/BSA)

These results indicate the neuroprotective property of the test drug which is helpful in treating memory and cognitive deficits in AD (Table 6).

Table 6. Effect of dulaglutide on biochemical parameter.

Treatment group	Blood glucose (mg/dL)	ACh (μM/mg)	AChE (μM/mg)	Protein content (mg/g)	CK-BB (U/L)
Vehicle	87.507 ± 2.48	6.914 ± 4.67	0.589 ± 6.75	9.784 ± 4.11	243.45 ± 5.41
Diabetic control (D)	166.625 ± 7.64*	3.709 ± 6.66*	1.456 ± 8.09*	12.104 ± 6.31*	621.67 ± 5.42*
Alzheimer's control (A)	98.26 ± 3.47*	1.653 ± 6.54*	1.598 ± 6.76*	17.927 ± 5.29*	660.61 ± 7.16*
D+A	163.63 ± 5.18 [#]	0.893 ± 7.32 ^{*\$}	1.904 ± 4.51 ^{*\$}	23.456 ± 5.55 ^{#\$}	691.32 ± 8.41 ^{#\$}
D+A +standard (3mg/BSA)	146.25 ± 6.35 ^{#a}	7.922 ± 8.61 ^{\$a}	0.511 ± 4.27 ^{\$a}	9.112 ± 6.07 ^{\$a}	223.89 ± 7.66 ^{\$a}
D+A +test drug (1.5mg/BSA)	114.65 ± 8.87 ^{#c}	7.321 ± 7.52 ^{ac}	0.593 ± 5.83 ^{ac}	8.907 ± 7.18 ^{ac}	219.57 ± 9.23 ^{ac}

Note: Values were represented as mean ± SEM (n=9). Statistical analysis of data was performed using One-way ANOVA followed by post-hoc Tuckey's test. *p<0.05 when compared with normal, #p<0.05 when compared with diabetic control, \$p<0.05 when compared with Alzheimer's control, #a p<0.05 when compared with D+A control, #c p<0.05 when compared with standard.

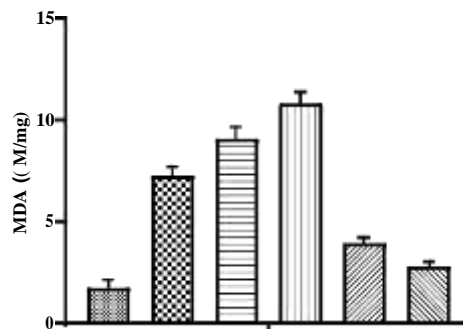
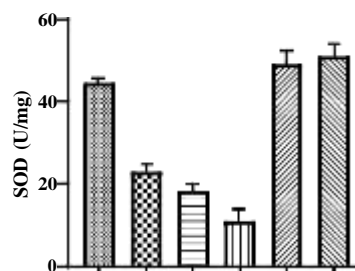
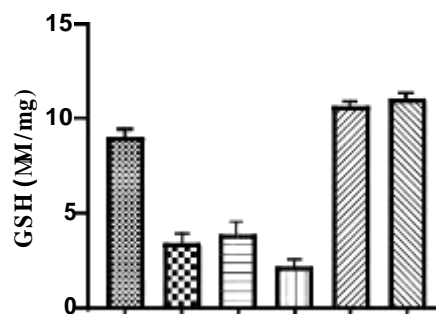
Tissue anti-oxidant parameters

Anti-oxidant parameters were estimated in different groups (Table 7) where lipid peroxidation was found to be increased when compared with vehicle control (1.75 ± 6.87) in diseased groups with 7.25 ± 8.31 in diabetic control, 9.09 ± 11.24 in Alzheimer's control and 10.81 ± 9.47 in D+A group. This was significantly reduced in standard and treatment groups with 3.94 ± 6.54 and 2.78 ± 7.69 respectively (Figure 10). SOD levels were decreased in diseased groups when compared with vehicle control (44.41 ± 9.86) having 22.80 ± 8.06 in diabetic control, 18.45 ± 9.68 in Alzheimer's control and 10.97 ± 12.35 in D+A group. This was found to be improved in standard and test groups with 49.10 ± 11.21 and 51.14 ± 10.42 respectively (Figure 11). Reduced glutathione levels were decreased in diseased groups with 3.47 ± 9.26 in diabetic control, 3.90 ± 11.45 in Alzheimer's control and 2.24 ± 11.11 in D+A group when compared to vehicle control with 9.04 ± 8.64 . Test drug treatment and standard group shown increase in these levels with 11.09 ± 10.68 and 10.67 ± 10.24 respectively (Figure 12). Catalase activity was found to be lowered in diseased groups with 0.61 ± 10.55 in diabetic control, 0.47 ± 10.89 in Alzheimer's control and D+A group with 0.20 ± 9.37 when compared with vehicle control having 1.03 ± 8.75 . This activity was enhanced in treatment group and standard group with 1.12 ± 11.75 and 1.02 ± 11.52 respectively (Figure 13). Improvement in anti-oxidant parameters indicates the neuroprotective effect of the test drug in experimental animals which can be used as a basis for the treatment of AD.

Table 7. Effect of dulaglutide on anti-oxidant parameters

Treatment groups	LPO ($\mu\text{M}/\text{mg}$)	ACh ($\mu\text{M}/\text{mg}$)	Reduced GSH ($\mu\text{M}/\text{mg}$)	Catalase ($\mu\text{M}/\text{mg}$)
Vehicle	1.75 \pm 6.87	44.41 \pm 9.86	9.04 \pm 8.64	1.03 \pm 8.75
Diabetic control (D)	7.25 \pm 8.31 [*]	22.80 \pm 8.06 [*]	3.47 \pm 9.26 [*]	0.61 \pm 10.55 [*]
Alzheimer's control (A)	9.09 \pm 11.24 [*]	18.45 \pm 9.68 [*]	3.90 \pm 11.45 [*]	0.47 \pm 10.89 [*]
D+A	10.81 \pm 9.47 ^{#S}	10.97 \pm 12.35 ^{#S}	2.24 \pm 11.11 ^{#S}	0.20 \pm 9.37 ^{#S}
D+A +standard (3mg/BSA)	3.94 \pm 6.54 ^a	49.10 \pm 11.21 ^a	10.67 \pm 10.24 ^a	1.02 \pm 11.52 ^a
D+A +test drug (1.5mg/BSA)	2.78 \pm 7.69 ^{ac}	51.14 \pm 10.42 ^{ac}	11.09 \pm 10.68 ^{ac}	1.12 \pm 11.75 ^{ac}

Note: Values were represented as mean \pm SEM (n=9). Statistical analysis of data was performed using One-way ANOVA followed by post-hoc Tuckey's test. ^{*}p<0.05 when compared with normal, [#]p<0.05 when compared with diabetic control, ^Sp<0.05 when compared with Alzheimer's control, ^ap<0.05 when compared with D+A control, ^cp<0.05 when compared with standard.

**Figure 10.** Representation of effect of dulaglutide on Lipid peroxidation. **Note:** (▨) Control, (▩) Diabetic Control, (▧) Alzheimer's control, (▦) Diabetic+ Alzheimer's control, (▥) Donepezil (3 mg/BSA), (▤) Dulaglutide (1.58 mg/BSA)**Figure 11.** Representation of effect of dulaglutide on SOD. **Note:** (▨) Control, (▩) Diabetic Control, (▧) Alzheimer's control, (▦) Diabetic+ Alzheimer's control, (▥) Donepezil (3 mg/BSA), (▤) Dulaglutide (1.58 mg/BSA)**Figure 12.** Representation of effect of dulaglutide on reduced GSH. **Note:** (▨) Control, (▩) Diabetic Control, (▧) Alzheimer's control, (▦) Diabetic+ Alzheimer's control, (▥) Donepezil (3 mg/BSA), (▤) Dulaglutide (1.58 mg/BSA)

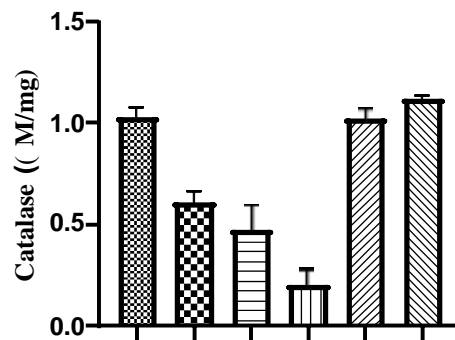


Figure 13. Representation of effect of dulaglutide on catalase. **Note:** (■) Control, (▨) Diabetic Control, (▩) Alzheimer's control, (▧) Diabetic+ Alzheimer's control, (▦) Donepezil(3 mg/BSA), (▥) Dulaglutide(1.58 mg/BSA)

Histopathological examination

Congo red staining was used to observe the amyloid beta plaque deposition in hippocampal region of the brain. Amyloid beta plaque deposition was observed in all the diseased control groups with severe deposition in Diabetic-Alzheimer's disease control group and moderate plaque deposition in diabetic control and Alzheimer's disease control. Treatment with dulaglutide (1.5 mg/BSA) did not completely eradicate the amyloid plaque but significantly presented a protective effect with mild to moderate plaques when compared with the diseased control groups. Treatment group also show corresponding results with the standard donepezil hydrochloride group presenting a normal histology with the control group (Figure 14).

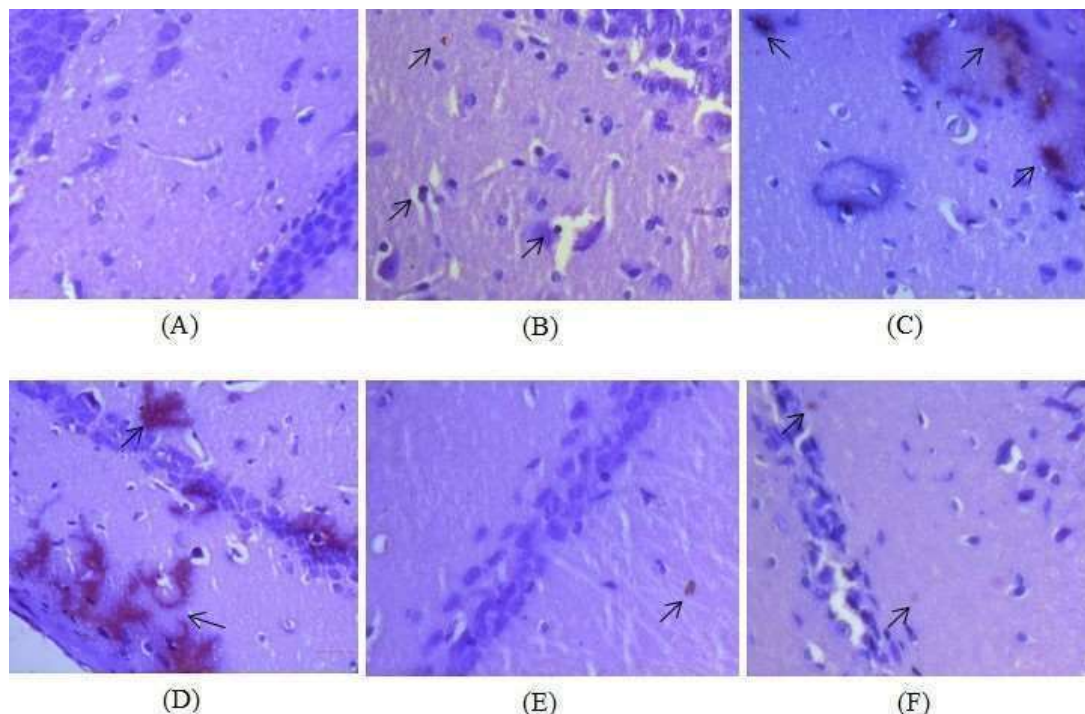


Figure 14. Histopathological examination of hippocampus with Congo red stain. (A) Group 1: Control group showing absence of plaque in the hippocampus. (B) Group 2: Diabetic control group treated with 30 mg/kg STZ and 120 mg/kg NA showing mild to moderate plaque deposition in the hippocampus. (C) Group 3: Alzheimer's disease control group treated with 100 mg/kg $AlCl_3$ for 42 days showing moderate plaque deposition in the hippocampus. (D) Group 4: Diabetic and Alzheimer's disease control group treated with 30 mg/kg STZ and 120 mg/kg NA followed by 100 mg/kg $AlCl_3$ for 42 days showing severe plaque deposition in the hippocampus. (E) Group 5: Standard group treated with 30 mg/kg STZ and 120 mg/kg NA+100 mg/kg $AlCl_3$ along with 3 mg/BSA donepezil hydrochloride for 42 days showing decrease in the intensity of the plaque corresponding to control group. (F) Group 6: Received 30 mg/kg STZ and 120 mg/kg NA+100 mg/kg $AlCl_3$ with 1.5 mg/BSA dulaglutide weekly for 42 days as treatment showing protective effect against plaque with mild deposition and corresponding histology of control group.

H&E staining was used for the microscopic examination of the hippocampal tissues of brain. Diabetic-Alzheimer's disease control group showed increased number of degenerated neurons with occasional plaques whereas; the diabetic control and Alzheimer's disease control groups shown occasional neuronal degeneration. Treatment with dulaglutide (1.5 mg/BSA) has significantly decreased the degeneration when compared with the diseased control groups and appeared more or less likely with the standard donepezil hydrochloride group and control group indicating its neuroprotective effect (Figure 15).

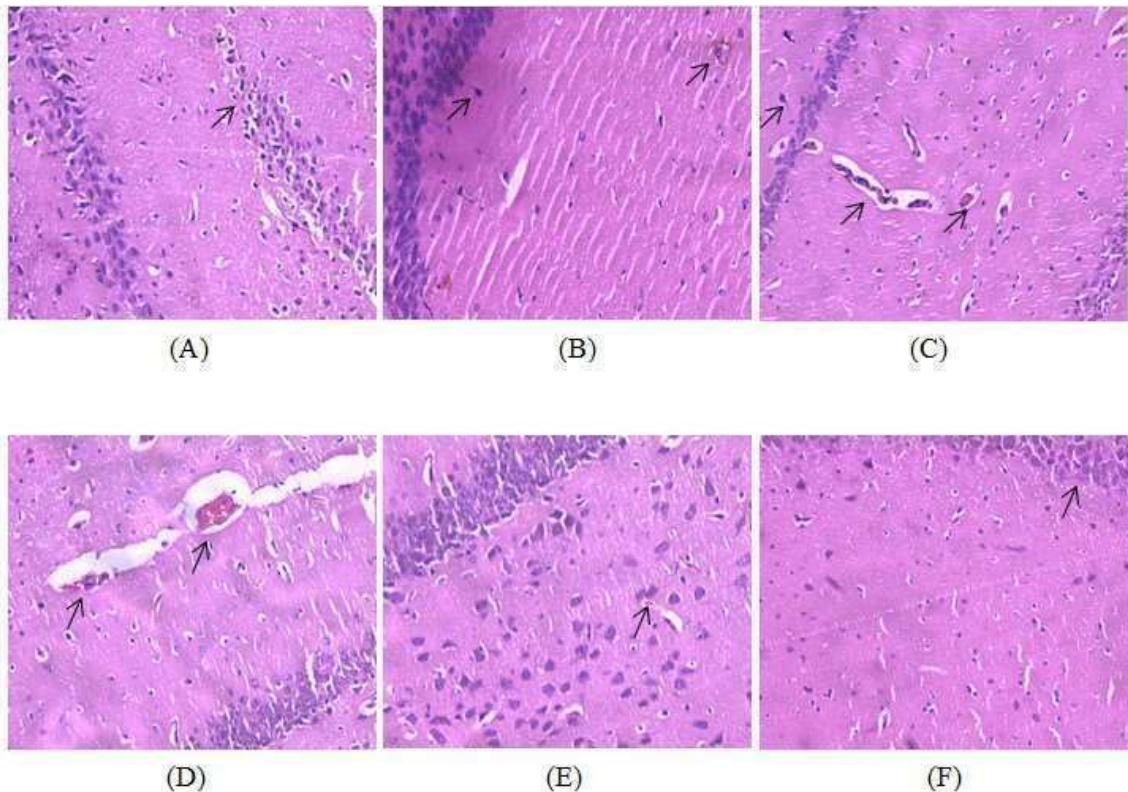


Figure 15. Histopathological examination of H and E stained hippocampus. (A) Group 1: Control group showing normal histology of the hippocampus with no signs of neuronal degeneration. (B) Group 2: Diabetic control group treated with 30 mg/kg STZ and 120 mg/kg NA showing occasional degeneration of neurons when compared to control group. (C) Group 3: Alzheimer's disease control group treated with 100 mg/kg $AlCl_3$ for 42 days showing occasional degeneration of neurons when compared with control group. (D) Group 4: Diabetic and Alzheimer's disease control group treated with 30 mg/kg STZ and 120 mg/kg NA followed by 100 mg/kg $AlCl_3$ for 42 days showing increased number of degenerated neurons with occasional plaques. (E) Group 5: Standard group treated with 30 mg/kg STZ and 120 mg/kg NA+100 mg/kg $AlCl_3$ along with 3mg/BSA donepezil hydrochloride for 42 days showing less number of degenerated neurons when compared to diseased groups. (F) Group 6: Received 30 mg/kg STZ and 120 mg/kg NA+100 mg/kg $AlCl_3$ with 1.5mg/BSA dulaglutide weekly for 42 days as treatment showing protective effect in the hippocampus against neuronal degeneration with few degenerated neurons.

CONCLUSION

This work was performed to evaluate the neuroprotective effect of dulaglutide against cognitive dysfunction (induced with $AlCl_3$) in Alzheimer's associated Type-2 diabetic rats. From the results obtained from behavioral studies it was indicative that the test drug used in the treatment was found to be effective in improving cognition and memory in Alzheimer diabetic rats. Also, the test drug has lowered blood glucose levels exhibiting its anti-diabetic effect. Results obtained from biochemical parameters indicates the effectiveness of the test drug in improving acetylcholine levels and anti-oxidants and also by reducing acetylcholinesterase levels, protein content, CK-BB and lipid peroxidation. Histopathological examination also revealed that treatment with dulaglutide caused an appreciable decrease in the amyloid beta plaque deposition (observed under Congo red stain) and also decreased the degeneration of neurons (observed under H and E stain) in the hippocampus of brain suggesting its neuroprotective effect. Neuroprotective

effect of GLP-1 analogues dulaglutide was produced because of the existence of GLP-1 receptors which is a potential target for GLP-1 and its analogues thereby presenting cognitive benefits. It can be a drug of choice in AD as it is an easily available drug with minimum side effects and effective at lower doses. Hence, dulaglutide can be employed in the treatment of Alzheimer's disease alone and in Type-2 diabetic Alzheimer's patients. Long term treatment with dulaglutide may help in ameliorating the severity of neurodegeneration and lessens the complications associated with diabetes which in turn promotes the life expectancy of patients.

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