Induction of Type 3 Diabetes mellitus by the Administration of Smaller Doses of Aluminium Chloride to the STZ Pretreated Rats

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Abstract

The present study is designed to develop Type-3 diabetes mellitus characterized by insulin resistance, mainly at the brain level. The experimental rats in *group-1:* received vehicle (5 ml/kg, i.p.) for 28 days, *group-2-4:* received aluminium chloride ($AlCl_3$) (12.5, 25, and 50 mg/kg, i.p.) daily for 28 days, *group-5:* received single dose of STZ (45 mg/kg, i.p.), and *group-6-8:* received STZ and $AlCl_3$ (12.5, 25, and 50 mg/kg, i.p. daily for 28 days) from the third day of STZ administration. The behavioral analysis was initiated on the 29th day, estimating locomotor activity using an open field test, learning and memory-related functions using elevated plus maze (EPM) and morris water maze (MWM) tests. Afterwards, the rats were sacrificed, and brain and blood were collected for whole-brain neurochemical and plasma biochemical assays, including glucose, insulin, nitrite, MDA and amyloid-beta levels. The brain tissues were sectioned, followed by H&E and congo red staining for histopathological examination. The results obtained demonstrated that the STZ pretreatment and $AlCl_3$ (12.5 mg/kg) treatment significantly impaired the cognition in EPM and MWM. Also, the STZ pretreatment and $AlCl_3$ (12.5 mg/kg) treatment significantly increased the brain glucose levels, brain insulin levels, lipid peroxidation and amyloid levels, suggesting the development of hyperinsulinemia, insulin resistance and amyloid pathology. Further, STZ pretreatment and $AlCl_3$ (12.5 mg/kg) treated rats produced the marked degenerated neurons, neuronal loss, and the marked deposition of amyloid fibrils suggesting the development of neurodegenerative changes. In conclusion, the present findings suggested that administering low doses of $AlCl_3$ to the STZ pretreated experimental rats results in the development of marked hyperinsulinemia, insulin resistance, cognitive impairment, amyloid-beta deposition, and neuronal degeneration, reflects the features of Type-3 diabetes mellitus (T3DM).

1.0 Introduction

Insulin is a peptide hormone secreted by the β cells of pancreas responsible for the regulation of blood glucose levels, cellular glucose uptake, metabolism of carbohydrates, lipid and protein metabolism, cell division, and growth (Wilcox 2005). The condition where the insulin is not able to function normally despite the presence of either the normal or increased level of insulin is called insulin resistance. Due to the major role of insulin in the regulation of glucose, impaired insulin sensitivity is always explained in the context of its failure to reduce the blood glucose level both at normal and higher concentrations (Cefalu 2001; Reaven 2004). Thus, the systemic insulin resistance is mainly characterized by the reduced clearance of glucose in response to insulin (Reaven 1988) and may result in the consequences including hypertension, dyslipidemia, visceral adiposity, hyperuricemia, inflammation, inflammatory markers, endothelial dysfunction, etc. (Freeman and Pennings 2021). Insulin resistance resulted in the compensatory increase in insulin production and release further resulting in the development of hyperinsulinemia (Freeman and Pennings 2021).

Hyperinsulinemia is defined as a condition characterized by altered insulin secretion or dysregulated insulin clearance that results in raised levels of insulin levels without causing hypoglycemia.

Hyperinsulinemia occurs in obesity, diabetes, and metabolic disorders. It has been reported that hyperinsulinemia is more prevalent than insulin resistance in patients with obesity but without diabetes (Thomas et al. 2019). Hyperinsulinemia causes the primary insulin resistance mainly by the downregulation of its downstream signaling pathway or by the disruption of beneficial factors secreted from adipocytes responsible for the stimulation of insulin secretion resulting in hyperinsulinemia (Czech 2017). It has been reported that hyperinsulinemia mediated higher insulin secretion independent of insulin sensitivity (Mari et al. 2011). Hyperinsulinemia is known to confer various deleterious effects including impaired insulin signaling, mitochondrial abnormality, oxidative stress, calcium dyshomeostasis, sympathetic nervous system dysfunction, alterations in immune response, etc (Bzotte et al. 2014). Further, hyperinsulinemia is found to be responsible for neurodegenerative changes (Xu et al. 2004). Hence, hyperinsulinemia is more likely a primary defect as compared to compensatory insulin resistance (Thomas et al. 2019). Further, hyperinsulinemia has been implicated in the pathogenesis of type 2 diabetes, obesity, chronic inflammation, hypertriglyceridemia, and Alzheimer's disease (Kopp 2019).

In preclinical studies the streptozotocin (STZ) alone or together with high-fat diet (HFD) or in combination with NAD is mainly used to induce type 2 diabetes (Furman 2015; Reed et al. 2000; Srinivasan et al. 2005) characterized by the hyperglycemia, progressive insulin resistance and hyperinsulinemia (Moharir et al. 2020; Ordóñez et al. 2007). Administration of STZ has been shown to exhibit cognitive impairment in the morris water maze (MWM) test (Li et al. 2016) and the other deleterious effects including oxidative stress, cholinergic deficits, and neurodegeneration (Chen et al. 2014). The cognitive impairment and neurodegenerative changes imposed by the STZ treatment mainly reflect the impairment of insulin signaling at the brain or neuronal level. Further, the administration of the diabetogenic dose of STZ is known to induce memory-related impairment in the experimental animals (Wang et al. 2022). However, the main limitations of these studies are that they did not tell anything about the change in the insulin levels in the brain and plasma, amyloid-beta (Aβ) levels, and the mechanism by which the cognitive impairment occurs in these animals. The cognitive impairment in the experimental animals is induced by the administration of aluminum (neurotoxic metal) that accumulates in the hippocampus and frontal cortex and causes impairment of spatial learning and memory (Struys et al. 1997). It has been reported that the chronic aluminum exposure in experimental animals decreases the performance in the radial arm maze (Abdel et al. 2011), water maze (Nampoothiri et al. 2015), and step-down test (Abdel et al., 2011), promote the production of Aβ (Exley et al. 1993; Luo et al. 2009; Mantyh et al. 1993; Sakamoto et al. 2006) and inhibit the brain-derived neurotrophic factor (BDNF) signaling in the brain (Ghribi et al. 2001; Huat et al. 2019; Johnson and Sharma 2003; Kawahara 2010). Aluminium chloride has been shown to induce hyperglycemia and insulin resistance in experimental animals (Chary et al. 2017). These findings suggested the similarity in the mechanism of aluminium and the STZ but the former is known to induce the cognitive impairment whereas the later is known to induce diabetes and insulin resistance in the experimental animals. Further none of the agents at the subdiabetogenic or diabetogenic dose has been shown to induce hyperinsulinemia in the brain.

Therefore, the present study is designed to develop the type-3 diabetes mellitus characterized by the presence of hyperinsulinemia and insulin resistance mainly at the brain level.

2.0 Materials And Methods

2.1 Animals

Wistar rats were acquired from DFSAH, LUVAS, Hisar, Haryana (India). Animals were kept at Central Animal House of Maharshi Dayanand University, Rohtak, under self-controlled environment of ambient temperature maintained at 25 ± 2°C, relative humidity at 60 ± 10%, and 12-12h light-dark cycle and water and food *ad libitum*. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) (Approval No.: 153-165 dated 17/12/2018).

2.2 Drugs and Treatments

Streptozotocin (STZ) was purchased from Sigma-Aldrich Co., India and Aluminium chloride ($AlCl_3$) from Loba Chemie, Maharashtra, India. STZ was freshly dissolved in citrate buffer (pH 4.4). Aluminium chloride was dissolved in distilled water. All the drugs were administered through the intraperitoneal (i.p.) route in a dose of 5 ml/kg.

2.3 Assessment of Locomotor activity in rats

2.3.1 Open field test (OFT)

Locomotor activity was assessed to confirm that behavioral changes were not due to any change in locomotor activity (Rajasankar et al. 2009). The rat was placed inside the square box of 80 × 80 × 40 cm in dimension and the floor was divided into 16 equal squares. Each rat was placed in the centre region and allowed to explore the open field for 5 min. Number of explored squares/crossings, and time spent in central and peripheral squares were noted. The floor was cleaned after each rat was tested.

2.4 Assessment of memory-related alterations in rats

2.4.1 Elevated plus maze (EPM) test

EPM is made of two open arms of 50×10 cm that are crossed by two closed arms of 50×10 cm having a wall of 40 cm in height (Sharma and Kulkarni 1991). Both open and closed arms were connected with the central square. The plus maze was placed 50 cm above the ground. Each rat was gently placed at the corner of the open arm facing opposite to the centre. Transfer latency (TL) defined as the time taken by the rat to enter in the closed arm with all paws, was noted. The cut-off time of 90 s was fixed if a rat could not find the closed arm. After 90 s, rat was gently pushed into one of the closed arms. After 4 min of free exploration, each rat was sent back to its home cage. The second trial, also known as the retention trial was performed after 24 h of acquisition trial and transfer latency was noted (Dhingra and Kumar 2012; Kulkarni, 2012).

2.4.2 Morris Water Maze (MWM) Test

Water maze is used to investigate spatial memory and learning (Morris 1981). The circular water maze, painted black, with a dimension of 210 cm in diameter and 51 cm of height. The maze was divided into four equal quadrants and filled with water upto 35 cm and maintained at a constant temperature (25 ± 2°C). A black-painted platform of 10 cm in width and 28 cm height was placed 2 cm below the water surface in the target quadrant (Quadrant 4) and 2 cm below the water surface. The position of the platform was same during each day's trial. Trials for memory acquisition (60 s/trial) were conducted for each rat. Escape latency in each quadrant was recorded to a maximum of 60 s. Rat was placed in each quadrant for 60 s at an interval of 2 min and escape latency was noted and each rat was tested for four days at every 24 h interval. During each trial, the rat was freely allowed to find the platform. The rat was guided to the platform and given 20 s to stay there if it didn't get on the platform in the 60 s. Mean escape latency indicated acquisition. After removing the platform in the probe-trial phase on fifth day, the rat was allowed to probe the tank for 240 s. Time spent in the target quadrant was considered an indicator of memory (Vorhees and Williams 2006).

2.5 Biochemical and neurochemical estimations

2.5.1 Blood Collection

At the end of the experiment, animals were sacrificed, followed by the collection of blood. Blood was centrifuged for 15 min at 2500×g to collect serum for the estimation of insulin, glucose, nitrite and MDA level.

2.5.2 Tissue Collection

The brain was isolated and then weighed. Tissue homogenates (10% w/v) were prepared in ice-cold 0.1 M phosphate buffer of pH 7.4. Homogenates were then centrifuged at 10,000×g for 15 min. Aliquots of the supernatant so formed were separated and used for neurochemical estimation.

2.5.3 Glucose level assay

Glucose levels in the blood and brain were measured by GOD – POD Method using ERBA Glucose kit. Glucose reagent was mixed with test sample and incubated at 37°C for 15 min. Absorbance was read at 505 nm on biochromatic analyzer. Concentration of glucose was measured using the formula:

Glucose level= Absorbance of Test/Absorbance of Standard × Concentration of standard

2.5.4 Insulin assay

Blood and brain insulin levels were measured by chemiluminescent immunoassay using commercially available kit (Beckman Coulter). Sample was mixed with mouse monoclonal anti-insulin alkaline phosphatase conjugate and paramagnetic particles covered with mouse monoclonal anti-insulin antibody. While the conjugate responds with a separate antigenic location on the insulin molecule, insulin

binds to the antibody in the solid phase. Substance bound to the solid phase was placed in the magnetic field after incubation, while unbound substance was discarded. The vessel was then filled with the chemiluminescent substrate Lumi-Phos 530, and light produced in the reaction was monitored using luminometer. Light production was directly proportional to the insulin concentration in the sample.

2.5.5 Amyloid beta (Aβ) assay

Congo Red (CR) fluorescence assay was performed to estimate the Aβ levels. Before use, a fresh CR solution was run through a 0.2 µm filter in potassium phosphate buffer solution (5 mM) with NaCl (150 mM). The samples were vortexed for 15 s after the CR solutions were added to protein solution (100 µg/ml), resulting in a final concentration of 5 µM. The absorption spectrum was recorded from 400 to 700 nm on a UV spectrophotometer (Shimadzu-1800). The spectra of CR solutions containing protein were compared with CR solutions alone. The production of amyloid formations was indicated by a red shift of the absorption band towards 540 nm. (Meehan et al. 2004).

2.5.6 Nitrite assay

The mixture of supernatant and Greiss reagent was incubated at 37°C for 10 min and absorbance was read at 546 nm with spectrophotometer. The concentration of nitrite was determined from sodium nitrite standard curve (Green et al. 1982).

2.5.7 Malondialdehyde (MDA) estimation

The extent of lipid peroxidation was determined quantitatively by the method described by Wills (Wills 1966). The amount of MDA was measured by reaction with thiobarbituric acid using UV spectrophotometer and absorbance was read at 532 nm. The values were calculated using the molar extinction coefficient of the chromophore ($1.56 \times 105\ mol^{-1}cm^{-1}$).

2.5.8 Histological examination

Brain tissues were isolated and placed in 10% formaldehyde for 2 hours (Francis et al. 2008). The tissues were then placed in fresh formaldehyde solution for 24 hr followed by dehydrating the tissue using ethanol (90% for 1 hr), cleaning in xylene, and embedding in paraffin. A thin section of 5 µm thickness was cut using a microtome and mounted on a glass slide and then stained with Hematoxylin and Eosin (H& E) solution. Sections were then observed under a microscope at a magnification of 40X (Bancroft 2008).

For Congo Red staining, the brain tissues were fixed in Bouin's solution. Tissues were processed as per the standard procedure of fixing, dehydrating, impregnating, embedding, sectioning, and staining with Congo Red. Amyloid- deposition was observed under a microscope at a magnification of 40X (Puchtler et al. 1962).

2.5.9 Experimental protocol

Male wistar rats were used in the present study as experimental animals consisting of equal number of rats (n=8) in each group. Experimental group included: **Group-1:** Vehicle treated rats; **Group-2-4:** $AlCl_3$ (12.5-50 mg/kg, i.p for 28 days) treated rats; **Group-5:** STZ (45 mg/kg, i.p. single dose) treated rats; **Group-6:** STZ pretreatment + $AlCl_3$ (12.5 mg/kg, i.p for 28 days) treated rats; **Group-7:** STZ pretreatment + $AlCl_3$ (25 mg/kg, i.p for 28 days) treated rats and **Group-8:** STZ pretreatment + $AlCl_3$ (50 mg/kg, i.p for 28 days) treated rats. After 28 days of the $AlCl_3$ administration, the rats were subjected to the behavioral testing for the determination of cognitive dysfunctions. Afterwards, the rats were sacrificed and the brain and blood samples were collected for the biochemical, neurochemical and histopathological assays.

2.6 Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using GraphPad Prism software (version 9). Values were expressed as Mean ± S.E.M. Value of $P < 0.05$ was considered statistically significant.

3.0 Results

3.1. Effect on locomotor activity in open field test (OFT)

One-way ANOVA revealed no significant effect of various treatments on time spent in centre squares ($F_{7,56}$=1.657, P=0.1387), peripheral squares ($F_{7,56}$=2.284, P=0.405), and the number of crossings ($F_{7,56}$=3.758, P=0.0021) in rats (shown in Figure-2).

3.2 Effect on memory related functions in EPM and MWM tests

One-way ANOVA revealed the effect of various treatments on transfer latency in EPM on day 1 ($F_{7,56}$=11.77, P<0.001) and day 2 ($F_{7,56}$=14.51, P<0.001) in rats (shown in Figure-3). Tukey’s post hoc test revealed that a significant increase in the transfer latency of Day 2was observed by STZ treatment (P<0.01) as compared to its control whereas a significant reduction in the transfer latency was observed by $AlCl_3$ (50 mg/kg, i.p.) treatment (P<0.01). However, the administration of $AlCl_3$ (12.5 mg/kg, i.p.) in STZ pretreated rats significantly increased the transfer latency as compared to $AlCl_3$ (12.5 mg/kg, i.p.) alone treated rats (P<0.001).

One way ANOVA revealed the effect of various treatments on time spent in the Target Quadrant ($F_{7,56}$=10.85, P<0.001), and frequency of platform crossing ($F_{7,56}$=1.459, P=0.2007), and frequency of target quadrant crossing ($F_{7,56}$=1.743, P=0.1176) by rats in MWM test (shown in Figure-4). Tukey’s post hoc test revealed that the administration of $AlCl_3$ (12.5 and 25 mg/kg, i.p.) to the STZ pretreated rats significantly decreased the time spent in target quadrant as compared to its control. Further, administration of $AlCl_3$ (12.5 mg/kg, i.p.) to the STZ pretreated rats significantly decreased the time spent in target quadrant as compared to $AlCl_3$ (12.5 mg/kg, i.p.) alone treated rats (P<0.05).

The results obtained demonstrated the development of cognitive impairment in the STZ pretreated rats receiving $AlCl_3$ (12.5 mg/kg for 28 days).

3.3 Effect on Glucose and Insulin levels of rats

One-way ANOVA revealed the effect of various treatments on the blood glucose levels ($F_{7,56}$=28.20, P<0.001) and brain glucose levels ($F_{7,56}$=89.63, P<0.001) (shown in Figure-5). Tukey's post hoc test revealed a significant increase in the blood glucose level in STZ treated group (P<0.001) as compared to its control. A further significant decrease in blood glucose level was observed after administration of $AlCl_3$ (12.5, 25, and 50 mg/kg, i.p.) to STZ pretreated rats (P<0.001). In contrast, a significant increase was observed in brain glucose levels of $AlCl_3$ (12.5, 25, and 50 mg/kg, i.p.) treated rats as compared to their respective control (P<0.01) whereas a significant reduction was observed in brain glucose levels of STZ treated rats as compared to control (P<0.001). A significant increase in brain glucose level was observed in the STZ pretreated rats receiving of $AlCl_3$ (12.5 and 25 mg/kg, i.p.) (P<0.001) whereas a significant decrease was observed in the brain glucose level of STZ pretreated rats receiving $AlCl_3$ (50 mg/kg) (P<0.001) as compared to their control.

One-way ANOVA revealed the effect of various treatments on the brain insulin levels ($F_{7,56}$=30.49, P<0.001) and plasma insulin levels ($F_{7,56}$=8.613, P<0.001) (shown in Figure-6). Tukey's post hoc test revealed a significant decrease in the brain insulin level in $AlCl_3$ (25 mg/kg, i.p.) (P<0.01) and $AlCl_3$ (50 mg/kg, i.p.) treated group (P<0.001) as compared to its control. A further significant decrease was observed in brain insulin levels after administration of $AlCl_3$ (25 mg/kg, i.p.) and $AlCl_3$ (50 mg/kg, i.p.) to STZ pretreated rats (P<0.05; P<0.001 respectively). A significant increase in the brain insulin level after administration of $AlCl_3$ (12.5 mg/kg, i.p.) in STZ pretreated rats (P<0.001) as compared to $AlCl_3$ (12.5 mg/kg, i.p.) alone treated group. On contrary, a significant increase in plasma insulin levels was observed in STZ treated group as compared to its control (P<0.001) whereas a significant reduction was observed in plasma insulin levels after administration of $AlCl_3$ (50 mg/kg, i.p.) in STZ pretreated rats as compared to its control (P<0.001). A significant increase was observed in plasma insulin levels after administration of $AlCl_3$ (12.5 mg/kg, i.p.) in STZ pretreated rats as compared to $AlCl_3$ (12.5 mg/kg, i.p.) alone treated rats (P<0.05).

3.4 Effect on brain amyloid-beta (Aβ) level

One-way ANOVA revealed the effect of various treatments on the brain Aβ levels ($F_{7,56}$=43.67, P<0.001) (shown in Figure-7). Tukey's post hoc test revealed that the significant increase in the brain Aβ levels in $AlCl_3$ (12.5 and 25 mg/kg, i.p.) (P<0.01), $AlCl_3$ (50 mg/kg, i.p.) (P<0.05) and STZ (P<0.001) treated group as compared to its control. A further significant increase was observed in brain Aβ level after administration of $AlCl_3$ (12.5 and 50 mg/kg, i.p.) to STZ pretreated rats (P<0.001 respectively) as compared to their control.

3.5 Effect on nitrite and MDA level of rats

One-way ANOVA revealed the effect of various treatments on brain nitrite levels ($F_{7,56}$=11.34, P<0.001) and plasma nitrite levels ($F_{7,56}$=4.440, P=0.006) (shown in Figure-8). Tukey's post hoc test revealed that a significant increase in brain nitrite levels was observed after administration of $AlCl_3$ (50 mg/kg, i.p.) to STZ pretreated rats as compared to the control (P<0.05) whereas a significant decrease in plasma nitrite levels was observed in $AlCl_3$(12.5 mg/kg, i.p.) and STZ treated rats as compared to their respective control (P<0.05).

One-way ANOVA revealed the effect of various treatments on brain MDA levels ($F_{7,56}$=17.99, P<0.001) and plasma MDA levels ($F_{7,56}$=47.564, P<0.001) (shown in Figure-8). Tukey's post hoc test revealed that a significant decrease in brain MDA levels was observed in $AlCl_3$(12.5 mg/kg, i.p.) and STZ-treated rats as compared to their respective control (P<0.01) whereas a significant increase was observed in $AlCl_3$ 50 mg/kg treated rats as compared to their respective control (P<0.01). A significant increase was observed in $AlCl_3$ (12.5 and 25 mg/kg, i.p.) administered STZ pretreated rats (P<0.05; P<0.001) respectively as compared to their respective control. On contrary, an increase in plasma MDA levels in $AlCl_3$ (12.5 and 50 mg/kg, i.p.) levels was observed in their respective control (P<0.05 and P<0.01 respectively). Further, a decrease in plasma MDA levels was observed in $AlCl_3$(12.5, 25, and 50 mg/kg, i.p.) administered STZ pretreated rats as compared to their respective controls (P<0.001).

4.0 Discussion

In the present study, the administration of the $AlCl_3$ (25 and 50 mg/kg, i.p.) decreased the brain insulin and increased the plasma insulin level significantly as compared to the control rats but did not influence the plasma insulin level significantly as compared to the control. One previous study has suggested no significant reduction in hippocampal insulin in rats treated with $AlCl_3$ (50 mg/kg/day i.p for 6 weeks) as compared to control (Bazzari et al. 2019). Further, the administration of $AlCl_3$ (34 mg/kg/day, p.o. for 12 weeks) alone also did not significantly change serum insulin levels compared with normal rats (Ali et al. 2019). Administration of $AlCl_3$ (10 mg/kg, i.p.) for 30 days has significantly increased the serum insulin level and insulin resistance index on the 10^{th} and 20^{th} day of treatment, but serum insulin level reduced on day 30 due to the decrease in pancreatic β-cell function as a result of pancreatic damage that occurred with the progression of treatment (Wei et al. 2018). In the present study, brain and plasma insulin levels were determined after the 30 days of the administration of single-dose STZ (35 mg/kg, i.p.), and the results suggested a rise in the brain and plasma insulin levels as compared to control. However, the brain insulin level was not significantly increased as compared to the control. Various previous studies have reported the effect of the STZ on the brain and plasma insulin levels. The majority of the studies suggested a reduction in the plasma insulin level after the STZ administration due to the necrotic effect of STZ on the pancreatic beta cells. In one study, the administration of STZ (65 mg/kg, i.v.) significantly decreased the plasma insulin level as compared to the control group (Lin et al. 2018). Further another study suggested that the administration of STZ (50 mg/kg, i.p.) to the mice has

been shown to significantly reduced plasma insulin levels as compared to control (Hemmati et al. 2018). However, in the present study, we used the lower dose of STZ, and the blood glucose levels of the STZ-treated rats were found to be reduced on the 28^{th} and 30^{th} days of administration. This finding suggested either the regeneration of the beta cells or transformation of non-beta cells into beta cells or the degeneration of the survived beta cells which is responsible for the insulin release and the raised plasma insulin levels of the STZ-treated rats. This fact is supported by a previous study suggesting that after administering the STZ, cells that were not affected by STZ-mediated cell death have shown raised mitotic characteristics. Bonner-Weir et al. 1989 showed increased mitosis, cell death, and hypertrophy in cells. They further suggested that hypertrophy may be linked with increased β-cell mass. Apoptosis regulates the rate of mitosis, which may maintain the islet cell number. They suggested that increased β-cell mass might be due to replication, cellular hypertrophy, or islet neogenesis by ductal cell differentiation (Bonner-Weir 1994). Some authors have also suggested the regeneration mechanisms of β-cells indicating the cell trans-differentiation from non-β cells to insulin-producing cells (Bonner-Weir et al. 1989; Bonner-Weir 1994). β-cells were found to be damaged after four days of neonatal STZ induction. On the 8^{th} day, β-cell recuperation occurred. After 20 days of STZ injection, β-cell mass was decreased even after the blood glucose level returned to normal (Liang et al. 2011). In contrast to these facts, Havrankova et al. 1979 reported no change in brain insulin level and brain insulin receptors but depletion of insulin in blood and pancreas was observed after 30 days of diabetogenic dose of peripherally injected STZ. Whereas in the present study the administration of the lower dose of STZ by i.p. route increased the level of insulin in the brain and the plasma, Lozano et al. 2017 have suggested that upon the administration of the STZ (40 mg/kg, i.m.) the plasma insulin levels increased significantly as compared to control resulting in the development of insulinemia. Insulin resistance alters glucose disposal and results in a compensatory rise in hyperinsulinemia and insulin production by β-cells. Insulin resistance may cause hyperglycemia, hyperuricemia, dyslipidemia, visceral obesity, increased inflammatory markers, endothelial dysfunction, etc. (Freeman et al. 2021). Hyperinsulinemia is termed as increased circulating insulin to its usual level relative to blood glucose, which does not cause hypoglycemia. Hyperinsulinemia is further referred to as the altered insulin secretion and/or clearance that results in increased insulin without causing hypoglycemia and is found commonly in obesity and metabolic disorders. In obese patients not suffering from diabetes, hyperinsulinemia and increased insulin secretion are found more prevalently than insulin resistance. Hence, hyperinsulinemia and increased insulin secretion may contribute to insulin resistance (Ferrannini et al. 1997; Thomas et al. 2019). Insulin resistance is the major cause of the compensatory increase in beta-cell insulin production and hyperinsulinemia (Freeman et al. 2021).

Memory-related functions in the experimental animals are mainly determined using the EPM test and MWM test. In the present study the administration of $AlCl_3$ (12.5, 25, and 50 mg/kg. i.p.) daily for 28 days) alone did not significantly affect the transfer latency on day-1 and day-2 in the EPM test. Chronic administration of $AlCl_3$ (100 mg/kg p.o.) significantly elevated transfer latency in animals treated with $AlCl_3$ as compared to control indicating the memory deficits due to administration of $AlCl_3$ (Thippeswamy et al. 2013). In the elevated plus-maze test, the animals treated with $AlCl_3$ (100 mg/kg., i.p.) exhibited a significant increase in retention transfer latency as compared to the control group (Thenmozhi et al.

2017). $AlCl_3$ (100mg/kg for 42 days) showed an increase in the transfer latency showing impairment in memory retention (Dulla and Bindhu 2021). Further, no significant difference was observed in the transfer latency of day-1 and day-2. The administration of the STZ significantly increased the transfer latency on day-1 and day-2 in the EPM. However, no significant difference was observed between the transfer latency of day-1 and day-2. STZ (45 mg/kg, i.p.) induced diabetic rats significantly increased transfer latency on days 1 and 2 as compared to control (Tamaddonfard et al. 2013). However, the administration of a single dose of STZ followed by the administration of $AlCl_3$ (12.5 and 25 mg/kg, i.p. daily for 28 days) significantly increased the transfer latency on day-2 in the EPM test as compared to the $AlCl_3$ (12.5 mg/kg and 25 mg/kg, i.p daily for 28 days) alone treated rats.

The administration of a single dose of STZ followed by the administration of $AlCl_3$ (12.5 and 25 mg/kg, i.p. daily for 28 days) significantly increased the brain and plasma insulin levels significantly as compared to the STZ alone and $AlCl_3$ (12.5 mg/kg and 25 mg/kg, i.p daily for 28 days) alone treated rats.

In the present study, administration of $AlCl_3$ (12.5 and 25 mg/kg, i.p. daily for 28 days) alone did not affect time spent in the target quadrant, frequency of platform crossing, and frequency of target quadrant crossing. A previous study suggested an increase in the escape latency of $AlCl_3$ (10 mg/kg, i.p. daily for two months)-treated rats compared with the saline control group (Abdel-Salam et al. 2021). In the MWM test, $AlCl_3$ (50 mg/kg/day i.p for 6 weeks) during training days increased escape latency in comparison to the control group indicating the learning defects. In the probe trial, $AlCl_3$ increased the escape latency to reach the target quadrant and reduced the time spent in the target quadrant and the number of times rats crossed the target quadrant when compared to the control (Bazzari et al. 2019). Rats treated with $AlCl_3$ (75 mg/kg, p.o. for 60 days) exhibited a significant increase in escape latency time as compared to vehicle control rats (Attia et al. 2020). $AlCl_3$ (100 mg/kg for 60 days, p.o.) significantly increased the escape latency and time spent in the target quadrant (Elmorsy et al. 2021). In the present study, the administration of STZ to the rats did not affect the time spent in the target quadrant, frequency of platform crossing, and frequency of target quadrant crossing in the MWM test. Previous studies have suggested that in MWM, STZ (50 mg/kg, i.p.) treatment in mice, increased the escape latency (Hemmati et al. 2018). STZ (65 mg/kg, i.v.) administered group spent more time and distance in reaching the visible platform and lower swimming speedas compared to the vehicle group (but the difference was not statistically significant). STZ treated group spent more time and distance to reach the hidden platform. In the probe test on the 33rd day, STZ treated group spent less time spent in the target quadrant and had a shorter distance ratio (distance traveled in the target quadrant per total distance traveled) as compared to the vehicle-treated group. On the 34th day, the STZ group took a longer time and longer distance to reach the hidden platform in the acquisition and retrieval trials of working memory as compared to the vehicle-treated group (Lin et al. 2018). Cognitive impairment was induced by ICV injection of STZ (3 mg/kg, i.c.v.) as manifested in the water maze test. The results suggested that the training was delayed for the i.c.v. treated STZ mice as they took more time and longer distance to find the platform as compared to the

control group, indicating short-term memory impairment. Further, in the probe trial, STZ-treated animals showed a significant reduction in the time spent in the target quadrant as compared to the normal group (Halawany et al. 2017). The administration of a single dose of STZ followed by the administration of $AlCl_3$ (12.5 and 25 mg/kg, i.p. daily for 28 days) decreased significantly decrease the time spent in the target quadrant as compared to the $AlCl_3$ (12.5 and 25 mg/kg, i.p. daily for 28 days) alone treated rats.

The daily administration of $AlCl_3$ (12.5 mg/kg, for 28 days) to the STZ-treated rats increased the brain insulin level significantly as compared to the STZ-treated rats. Further brain insulin level of the $AlCl_3$ (25 and 50 mg/kg, i.p. for 28 days) and STZ treated rats was lesser than the STZ alone treated rats. The daily administration of $AlCl_3$ (12.5 and 25 mg/kg, for 28 days) to the STZ-treated rats increased the plasma insulin levels. Thus, the administration of $AlCl_3$ (12.5 mg/kg, i.p.) daily for 28 days to the STZ pretreated rats resulted in the development of insulinemia at the brain and peripheral levels which was accompanied by the memory-related alterations. Whereas the administration of $AlCl_3$ (12.5 mg/kg) to the STZ-treated rats increased the brain insulin level and plasma insulin level of STZ-treated rats. Thus, the results obtained demonstrated that the administration of low doses of aluminum chloride to the STZ-treated rats induces brain and plasma hyperinsulinemia.

The daily administration of $AlCl_3$ (12.5, 25, and 50 mg/kg, i.p. for 28 days) increased the level of Aβ in the brain of the rats as compared to the control. Further, the levels of Aβ decrease with the increases in the dose of $AlCl_3$ suggesting the lower dose have the Aβ promoting effects. Further brain insulin levels decrease with the increase in the dose of $AlCl_3$ suggesting the effect of the lower doses of $AlCl_3$ on the brain Aβ and insulin level. Various studies have suggested the effect of the $AlCl_3$ on the brain's Aβ level. One of the studies suggested that the administration of $AlCl_3$ (50 mg/kg/day i.p) markedly elevated the hippocampal $Aβ_{42}$ level (Bazzari et al. 2019), Whereas the other suggested that the administration of $AlCl_3$ (75 mg/kg, p.o. for 60 days) resulted in an increase in the protein levels of Aβ compared with normal control (Attia et al., 2020) and another study suggested the increase in the significant increase in Aβ(1–42) expression in the rats' cortex by the administration of $AlCl_3$ (175 mg/kg, p.o.) (Promyo et al. 2020). Administration of the STZ resulted in an increase in the level of Aβ and insulin in both blood and brain. Previously it has been reported that the administration of STZ (3 mg/kg, i.c.v.) to the mice showed significant increases in the concentration of ab in the control group (Halawany et al. 2017). The daily administration of $AlCl_3$ (12.5 and 25 mg/kg, i.p. for 28 days) to the STZ pretreated rats increased the levels of the Aβ more as compared to the STZ alone treated rats. Specifically, in the present study, daily administration of $AlCl_3$ (12.5 mg/kg, i.p. for 28 days) increased the levels of Aβ without significantly affecting the brain and plasma insulin level and memory-related behavior of rats in EPM and MWM tests. However, the daily administration of $AlCl_3$ (12.5 mg/kg, i.p. for 28 days) to the STZ pretreated rats resulted in a significantly increased in the level of the Aβ, insulin levels (both in brain and plasma), and impaired memory-related behavior in EPM and MWM as compared to the $AlCl_3$ (12.5 mg/kg, i.p. for 28 days) alone treated rats.

The daily administration of $AlCl_3$ (12.5, 25, and 50 mg/kg, i.p. for 28 days) significantly increased the brain glucose level as compared to the control group but did not affect the plasma glucose level. The previous findings suggested a significant increase in the blood glucose level upon the administration of ($AlCl_3$ 50 and 100 mg/kg, po for 28 days) (Chary et al. 2017). The administration of STZ significantly decreased the brain glucose and increased the blood glucose as compared to control. Various studies have reported that the administration of STZ increased blood glucose levels. But very few studies suggested the effect of STZ administration on the brain glucose level of the animals. One of the studies suggested that the administration of STZ (65 mg/kg, i.p.) increases the blood and brain glucose levels of rats as compared to control (Wang et al. 2012). Further daily administration of $AlCl_3$ (12.5 and 25 for 28 days) to the STZ pretreated rats was accompanied by the significantly increased brain glucose level (on the 30^{th} day) but did not affect the blood glucose level of rats as compared to control. One of the previous studies reported a significant increase in the blood glucose of the rats receiving the 2 doses of STZ (60 mg/kg) and $AlCl_3$ (50 mg/kg po for 28 days) as compared to the control (Chary et al. 2017). In the present study, no significant alteration in the blood glucose level was noted between the $AlCl_3$ (12.5 and 25 for 28 days) alone treated rats w.r.t STZ pretreated- $AlCl_3$ (12.5 and 25 for 28 days) treated rats. However, the plasma insulin level of the STZ pretreated- $AlCl_3$ (12.5 and 25 for 28 days) treated rats was significantly increased despite the normal blood glucose level. This reflects the development of hyperinsulinemia both at the brain and peripheral levels.

Administration of $AlCl_3$ (25 and 50 mg/kg, i.p. for 28 days) did not affect the brain nitrite level and the plasma nitrite levels of rats. However, the aluminium (12.5 mg/kg, i.p. for 28 days) treatment did not affect the brain nitrite levels but decreased the plasma nitrite levels significantly as compared to the control group. It has been reported that the administration of $AlCl_3$ (10 mg/kg, i.p. daily for 2 months) resulted in a significant increase in brain NO content (Abdel-Salam et al., 2021). Another study suggested that the nitrite level was noticeably increased in the $AlCl_3$ (50 mg/kg for 2 weeks) treated mice group (Al-Amin et al. 2019). Further, the STZ treatment increased the brain nitrite level but decreased the plasma nitrite level of rats significantly as compared to the control. A different study suggested different effects of STZ treatment on nitrite levels. For eg. According to one of the studies the administration of STZ (50 mg/kg i.p.) decreases the serum nitrite (Sharma and Singh 2011) whereas in another study the administration of STZ (50 mg/kg i.p.) significantly elevated in the brain of STZ (50 mg/kg, i.p.) treated animals as compared to the control group (Hemmati et al. 2018). The daily administration of $AlCl_3$ (12.5 and 25 mg/kg for 28 days) to the STZ pretreated rats significantly did not affect the brain and plasma nitrite level of $AlCl_3$ (12.5 and 25 mg/kg for 28 days) alone treated rats. Further, the administration of daily administration of $AlCl_3$ (50 mg/kg for 28 days) to the STZ pretreated rats significantly increased the brain nitrite level but did not affect the plasma nitrite level of rats as compared to the $AlCl_3$ (50 mg/kg for 28 days) alone treated rats

In the present study, the daily administration of $AlCl_3$ (12.5, 25, and 50 mg/kg for 28 days) increased the MDA levels in the brain in a dose-dependent manner. Further, the daily administration of $AlCl_3$ (12.5, 25,

and 50 mg/kg for 28 days) decreased the MDA levels in the brain in a dose-dependent manner. However, the daily administration of $AlCl_3$ (50 mg/kg for 28 days) increased the brain MDA level while daily administration of $AlCl_3$ (12.5 mg/kg for 28 days) increased the plasma MDA level significantly as compared to control. The administration of $AlCl_3$ (34 mg/kg/day, p.o.), caused significant increases in the MDA content of brain tissue homogenate compared with normal rats (Ali et al., 2019). In another study, the $AlCl_3$ (75 mg/kg, p.o.) treatment to the rats resulted in a significant elevation in the MDA levels suggesting the oxidation of polyunsaturated fatty acids of cell membrane resulting from excessive production of reactive oxygen species (Attia et al., 2020). In contrast, STZ administration reduced the brain MDA level but increased the plasma MDA levels significantly as compared to control. Previous reports suggested that the STZ (3 mg/kg, icv) treatment significantly increased the TBARS level as compared to the control (Rinwa et al. 2012). Further, the treatment of rats with STZ (50 mg/kg, i.p.) has been shown to increase the brain MDA level in comparison to the control group (Hemmati et al. 2018; Sharma and Singh 2011). The daily administration of $AlCl_3$ (12.5, 25, and 50 mg/kg for 28 days) to the STZ pretreated rats resulted in an increase in brain MDA level but decreased the plasma MDA level of rats significantly as compared to STZ alone treated rats. More specifically daily administration of $AlCl_3$ (12.5 mg/kg for 28 days) to the STZ pretreated rats did not result in a significant alteration in the brain and plasma MDA level as compared to $AlCl_3$ (12.5 50 mg/kg for 28 days) alone treated rats. However, the daily administration of $AlCl_3$ (25 mg/kg for 28 days) to the STZ pretreated rats increased the brain MDA level significantly as compared to the $AlCl_3$ (25 mg/kg for 28 days) alone treated rats. This might be due to the significant elevation in the MDA levels suggesting the oxidation of polyunsaturated fatty acids of cell membrane resulting from excessive production of reactive oxygen species (Attia et al. 2020).

5.0 Conclusion

It is concluded that the administration of low doses of $AlCl_3$ to the STZ-treated animals results in the development of hyperinsulinemia both at the peripheral and brain level. Brain insulin resistance (BIR) is defined as the inability of brain cells to respond to the insulin and is characterized by several manifestations including impaired neuroplasticity, impaired cognition and mood, neurotransmitter release, insulin metabolism, neuronal glucose uptake, increased production of ROS, impaired mitochondrial oxygen consumption, reduced ATP production, and mitochondrial dyshomeostasis inflammatory responses to insulin, etc. Since main cause for the emergence of the IR is the hyperinsulinemia responsible for the downregulation of insulin receptors and signaling pathways, the hyperinsulinemia in our study is responsible for the cognitive impairment as evident by the increase in the transfer latency on day 2 in EPM and MWM tests. Therefore, it is concluded that the development of hyperinsulinemia at the peripheral and brain level is responsible for the cognitive impairment and thus may be called as "Type-3-Diabetes mellitus" (T3DM).

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Author Contributions

Ms. Abhilasha Ahlawat: Collection of data and write the manuscript

Dr. Vaibhav Walia: Statistical analysis

Prof. Munish Garg: Study Design, Supervision and Proof Reading

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Figures

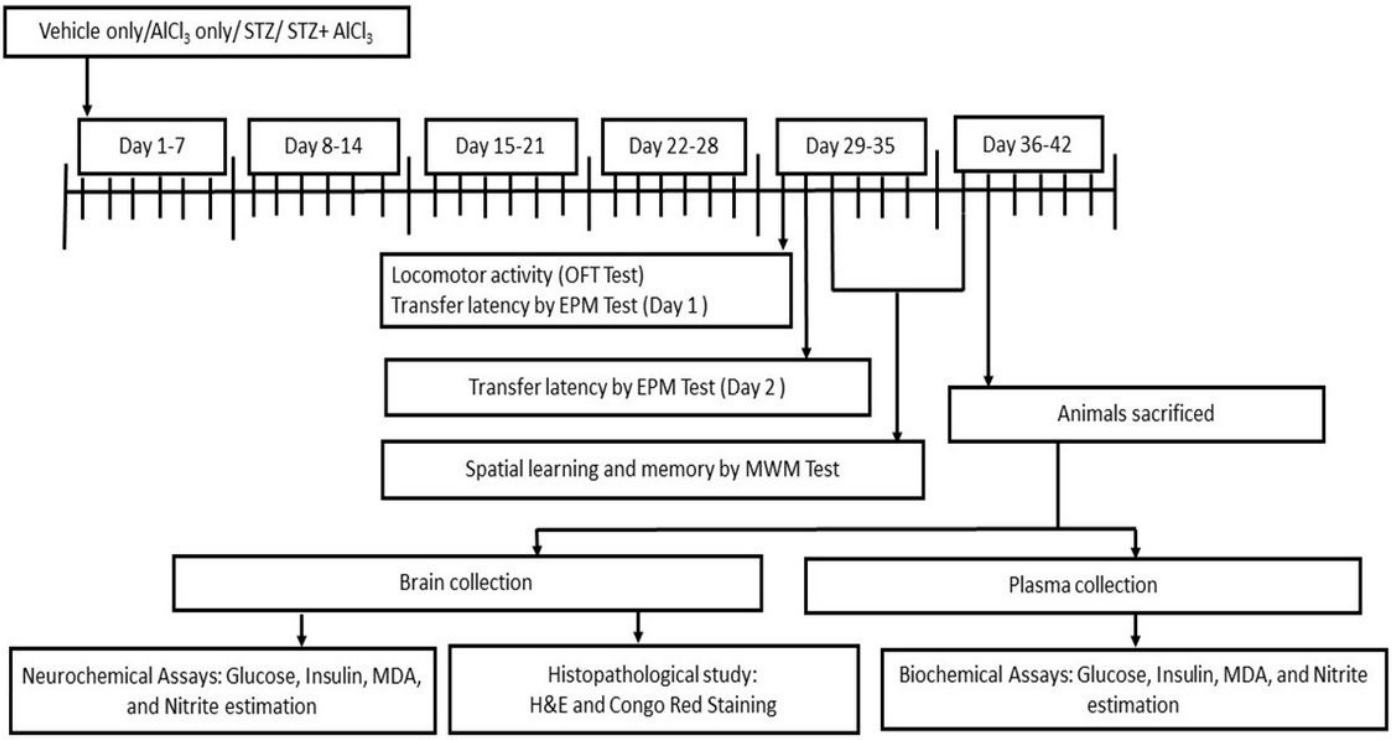

Figure 1

Experimental Protocol

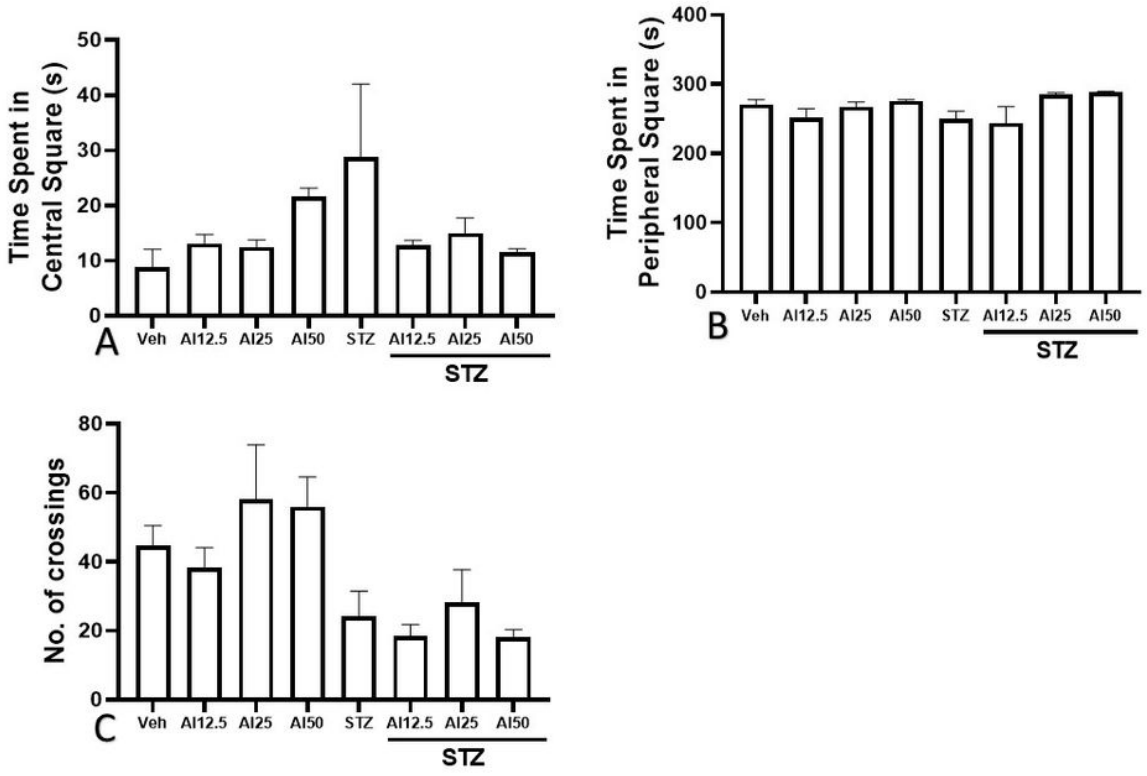

Figure 2

Effect of various treatments on the locomotor activity in OFT test.

Values are expressed as Mean±SEM, n=8 in each group.

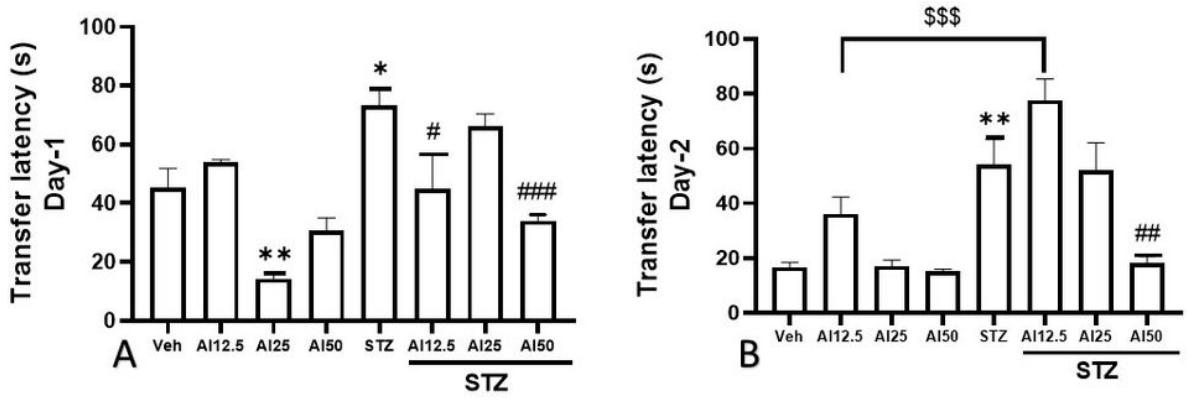

Figure 3

Effect of various treatments on the performance of rats in EPM test.

Values are expressed as Mean±SEM, n=8 in each group.

*P<0.05, **P<0.01, significant difference from the vehicle treated control group.

$^{\#\#}$P<0.01, $^{\#\#\#}$ P<0.001 significant difference from STZ treated rats.

$^{\$\$\$}$P<0.001 significant difference from $AlCl_3$ 12.5 mg/kg treated rats.

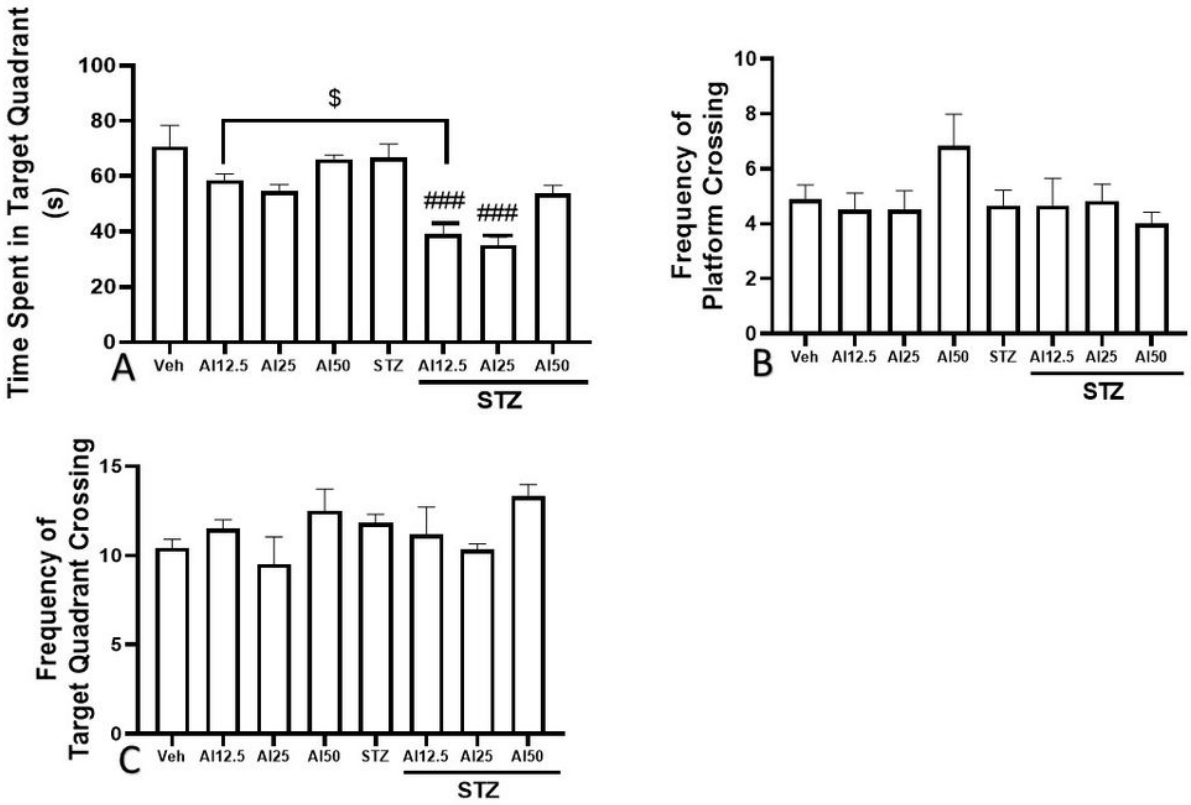

Figure 4

Effect of various treatments on the performance of rats in MWM test.

Values are expressed as Mean±SEM, n=8 in each group.

$^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ significant difference from STZ treated rats. $^{\$}P<0.05$, significant difference from $AlCl_3$ 12.5 mg/kg treated rats.

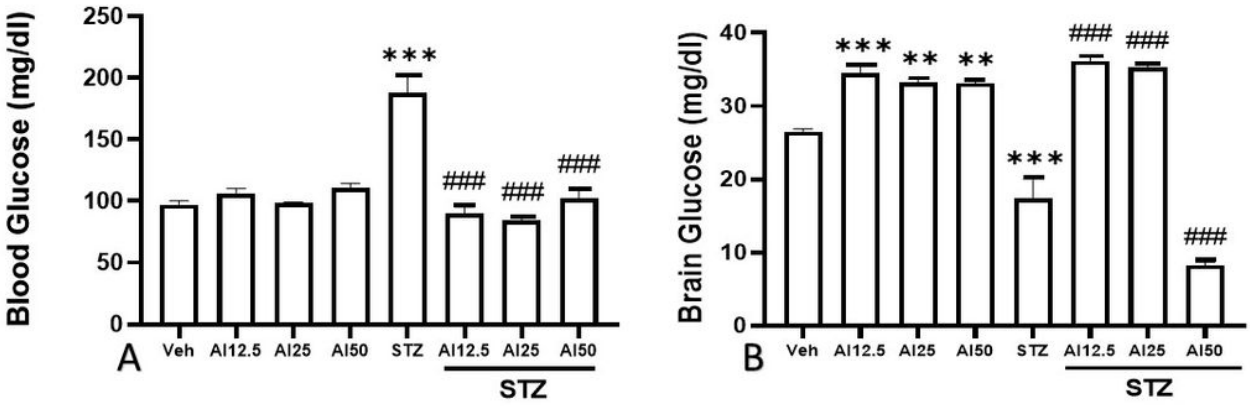

Figure 5

Effect of various treatments on the Blood glucose and Brain glucose levels of rats.

Values are expressed as Mean±SEM, n=8 in each group.

***P<0.001 significant difference from the vehicle treated control group.

P<0.001 significant difference from STZ treated rats.

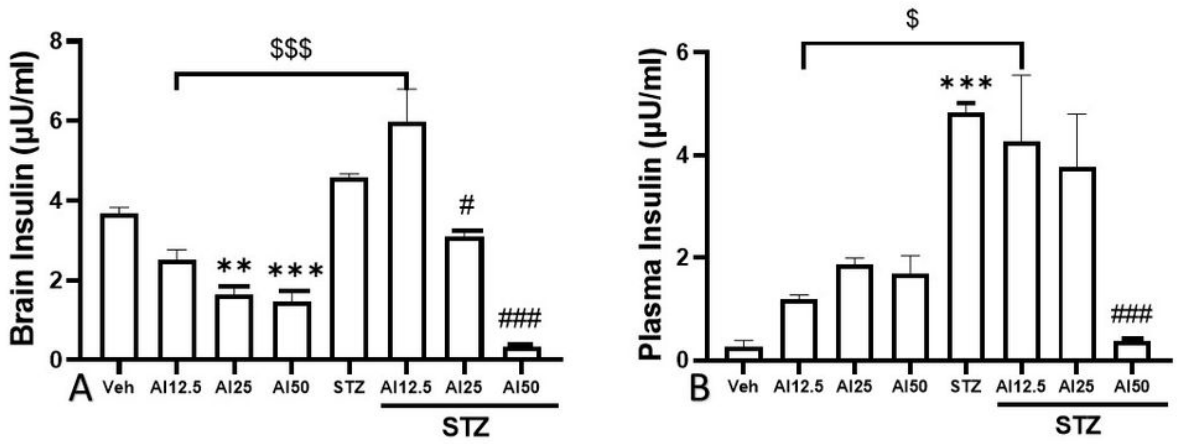

Figure 6

Effect of various treatments on Brain Insulin and Plasma Insulin Levels of rats.

Values are expressed as Mean±SEM, n=8 in each group.

**P<0.01, ***P<0.001 significant difference from the vehicle treated control group.

$^{\#}$P<0.05, $^{\#\#\#}$ P<0.001 significant difference from STZ treated rats.

$^{\$}$P<0.05, $^{\$\$\$}$P<0.001 significant difference from $AlCl_3$ 12.5 mg/kg treated rats.

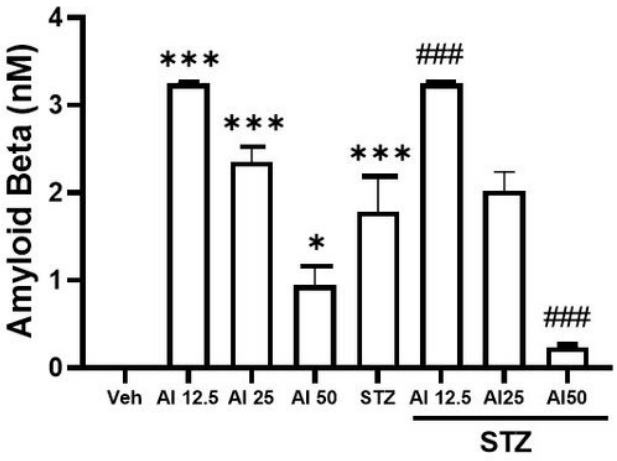

Figure 7

Effect of various treatments on Amyloid Beta Levels of rats.

Values are expressed as Mean±SEM, n=8 in each group.

*P<0.05, ***P<0.001 significant difference from the vehicle treated control group.

P<0.001 significant difference from STZ treated rats.

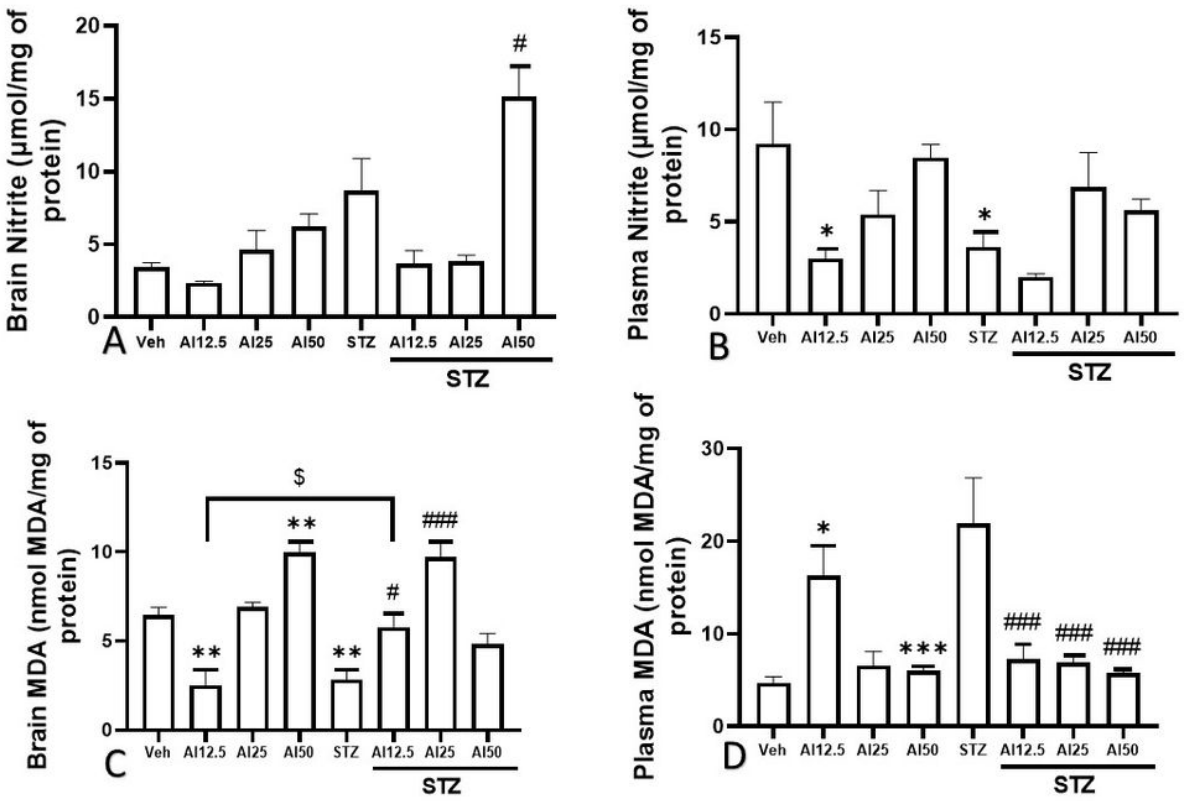

Figure 8

Effect of various treatments on Nitrite and MDA levels of rats.

Values are expressed as Mean±SEM, n=8 in each group.

*P<0.05, **P<0.01, ***P<0.001 significant difference from the vehicle treated control group.

$^{\#}$P<0.05, $^{\#\#\#}$ P<0.001 significant difference from STZ treated rats.

$^{\$}$P<0.05 significant difference from $AlCl_3$ 12.5 mg/kg treated rats.

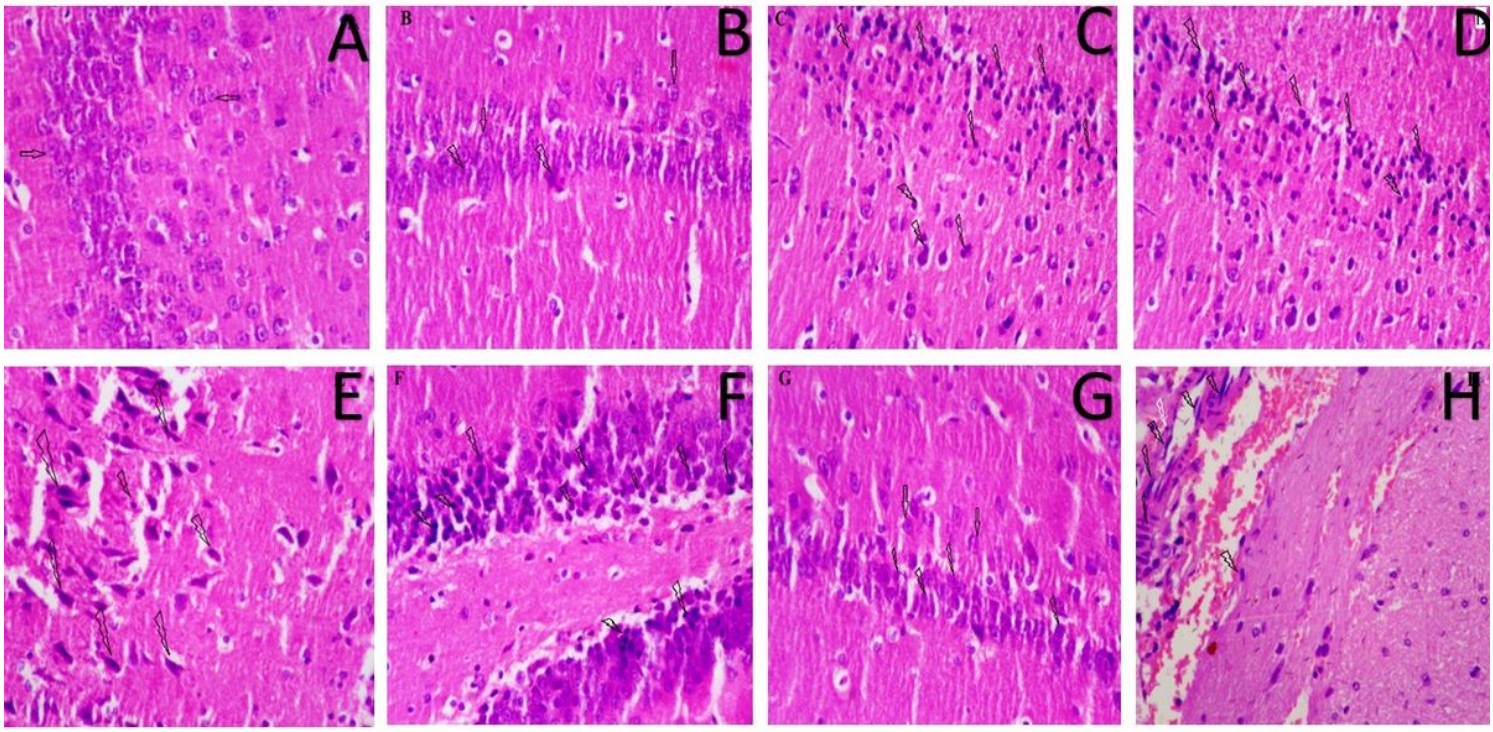

Figure 9

Histopathological evaluation of Rats Brain Section (H & E stained).

A: vehicle treated rats show normal healthy neurons.

B: $AlCl_3$12.5 mg/kg treated rats shows intact neurons with few dark stained scattered neurons with pyknotic nuclei.

C and D: $AlCl_3$ 25 and 50 mg/kg treated rats showed many dark stained, shrunken neurons having pyknotic nuclei showing marked degenerative neurons.

E: STZ 45 mg/kg treated rats showed dark stained degenerated neurons and marked neuronal loss.

F: STZ + $AlCl_3$ 12.5 mg/kg treated rats showed dark stained shrunken and degenerative neurons with significant pyknotic nuclei.

G: STZ+ $AlCl_3$ 25 mg/kg treated rats showed many dark stained degenerated neurons having pyknotic nuclei.

H: STZ + $AlCl_3$ 50 mg/kg treated rats showed marked degenerated neurons with significant pyknotic nuclei, marked neuronal loss and disorganized granular cell layer.

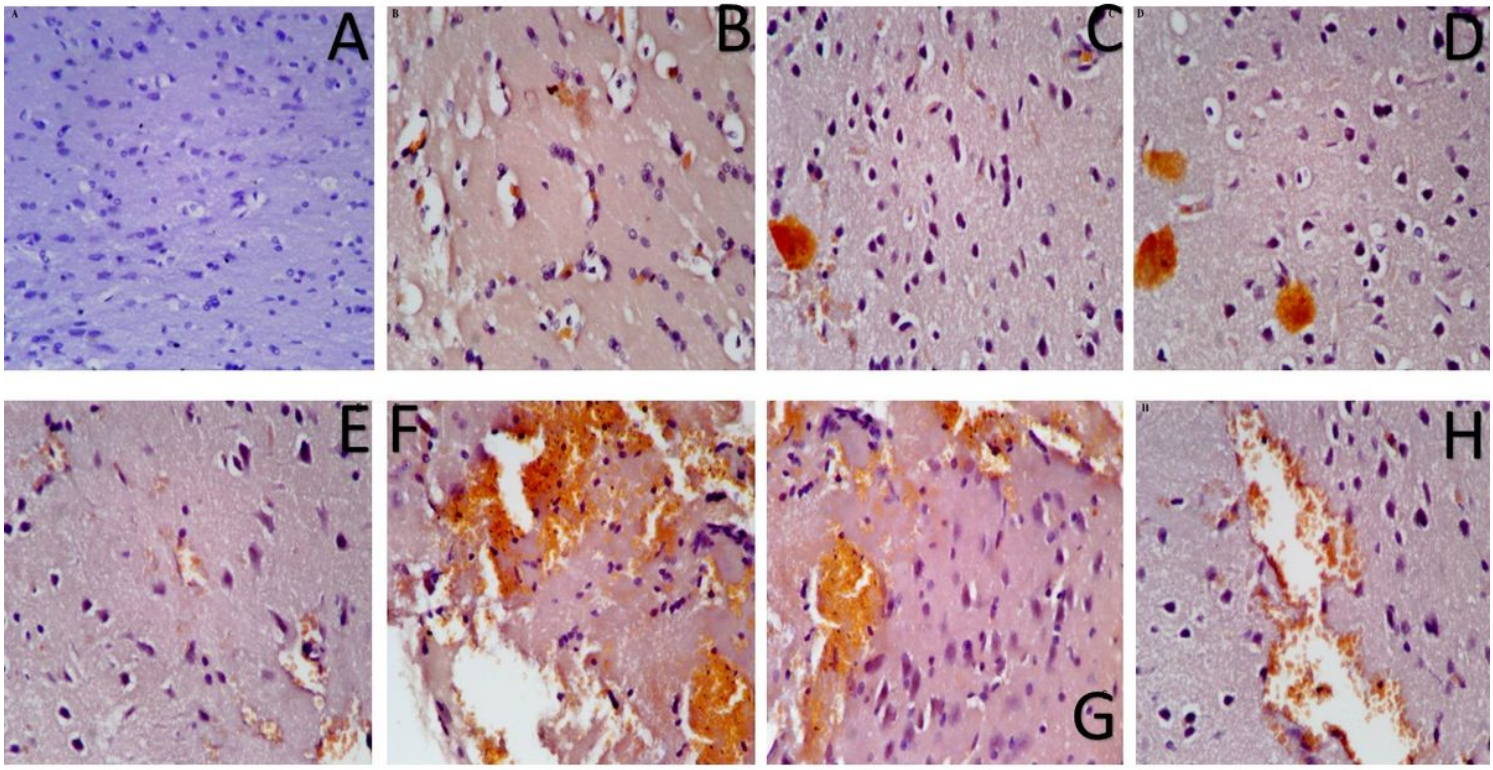

Figure 10

Histopathological study evaluation of Rats brain section (Congo Red staining).

A: Vehicle treated rats showed normal healthy neurons.

B, C, D & E: $AlCl_3$ (12.5 - 50 mg/kg) and STZ (45mg/kg) treated rats showed slight amyloid-beta deposition (orange red coloured deposits).

F, G & H: STZ pretreated + $AlCl_3$ (12.5 - 50 mg/kg) treated rats showed marked amyloid-beta deposition.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- graphicalabstract.jpeg