Beneficial effects of Insulin and Rivastigmine in Type-3 Diabetes mellitus in rats

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ABSTRACT

Type-3 diabetes mellitus (T3D) is a pathological condition that possesses the characteristics of both Diabetes and Alzheimer's disease, considered as brain diabetes. Treatment of T3D is still a challenging task for clinical practitioners. In this regard, this study was carried out to explore the effect of Insulin and Rivastigmine in the experimental rats. T3D was induced by administering streptozotocin (STZ; 35 mg/kg, i.p., single dose) followed by daily administration of aluminum chloride ($AlCl_3$) (12.5 mg/kg, i.p. × 28 days). The Insulin (1 IU s.c. daily × 28 days) and Rivastigmine (1 mg/kg, i.p. × 28 days) treatment was given 30 minutes prior to administration of $AlCl_3$. After 28 days of treatment, the rats were subjected to the estimation of various behavioral parameters using elevated plus maze (EPM) and Morris water maze (MWM) test and biochemical parameters including Insulin, glucose, malondialdehyde (MDA), nitrite, and amyloid beta (Aβ) levels. The results obtained revealed that the Insulin administration increased the square crossings in the open field, and reduced the transfer latency of T3D rats in the closed arm of EPM at day 2 and the frequency of platform crossing in the MWM test in T3D rats. The administration of Insulin and Rivastigmine reduced the blood glucose level and Aβ levels in the brain of T3D rats. Furthermore, Rivastigmine treatment also reduced the brain Insulin level of T3D rats. These studies indicate toward the beneficial effects of Insulin and Rivastigmine that open newer opportunities in the management protocol of T3D.

INTRODUCTION

Type-3 diabetes mellitus (T3D) is a pathological condition that resembles the characteristics of both type-1 and type-2 diabetes mellitus and is characterized by decreased Insulin production and Insulin resistance (IR), confined particularly to the brain, altering brain Insulin signaling, neurotoxins accumulation, and neuronal stress (Ashraf *et al.*, 2014; Caberlotto *et al.*, 2019; Nguyen *et al.*, 2020; Pasquier *et al.*, 2006; Rivera *et al.*, 2005; Sato *et al.*, 2006; Steen *et al.*, 2005). These manifestations affect basic activities such as learning and memory, thus, resulting in neurodegeneration (Nguyen *et al.*, 2020). T3D and Alzheimer's disease (AD) share common features including hyperinsulinemia, altered Insulin signaling cascade, and/or IR (Baker *et al.*, 2011; Weinstein *et al.*, 2019), aggregation of amyloid proteins such as Aβ and amylin (Cooper *et al.*, 1989; Hardy and Higgins, 1992; Lim *et al.*, 2010; Nguyen *et al.*, 2020) suggesting T3D, a neuro-endocrine disorder, characterized by dysregulated glucose homeostasis (GSK3β, IRS 1, Akt, altered Insulin signaling, Insulin receptor, and IGF 1 receptor), disturbed cell survival (impaired Bcl, Bad), altered lipid metabolism (APOE and JAK-STAT pathway), increased apoptosis (increased activity of caspase 3 and 8, altered p53 pathway) (Mittal *et al.*, 2016). Literature has indicated that the underlying Insulin signaling and/or IR are responsible for the emergence of memory and executive deficits (Ferreira *et al.*, 2018; Ormazabal *et al.*, 2018; Rorbach-Dolata and Piwowar, 2019) suggesting these as the ultimate causes of AD and related pathologies (Moloney *et al.*, 2010; Steen *et al.*, 2005; Talbot *et al.*, 2012).

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IR is responsible for tau hyperphosphorylation, and neuroinflammation leading to neurodegeneration (Kroner, 2009). Hyperinsulinemia impairs cognitive function causing cerebral microvascular and macrovascular damage (Den Heijer *et al.*, 2003). Both hyperinsulinemia and IR, are responsible for increasing inflammatory markers such as C-reactive protein and interleukin 6 (Hak *et al.*, 2001). Increased levels of these inflammatory markers result in the development and progression of AD. Sustained increase in the levels of inflammatory markers has been implicated in AD pathogenesis (McGeer and McGeer, 2004). Thus, hyperinsulinemia and IR, act as important factors that keep Insulin at the center stage of both diabetes and AD (Baker *et al.*, 2011). Further, hyperinsulinemia and/or IR may promote the secretion of butyrylcholinesterase (BuChE) (Randell *et al.*, 2005). BuChE and acetylcholinesterase (AChE) may also result in IR in AD (Houstis *et al.*, 2006; Randell *et al.*, 2005; Sharma *et al.*, 2006). Both, altered brain cholinergic activity and IR are known to accelerate the deposition of amyloid β in the AD brain (Das, 2007; Ho *et al.*, 2004; Reyes *et al.*, 2004).

Insulin plays an important role in the regulation of memory and learning. Insulin also regulates the concentrations of various neurotransmitters in the brain and affects long-term potentiation. Insulin receptors are located in the brain, particularly in the hippocampus, entorhinal, perirhinal, parahippocampal cortex, and medial temporal lobe areas (Zhao and Alkon, 2001). Other than being produced by neurons, Insulin may enter the brain by bypassing the blood–brain barrier (BBB) (Soto *et al.*, 2019). There are two different isoforms of the Insulin receptor: IR-A (predominantly present in the brain) and IR-B (predominantly present in peripheral tissues including the muscles, kidneys, and liver) (Mosthaf *et al.*, 1990). IR-A & B, both, are present in astrocytes while IR-A is exclusively present in neurons (Abbott *et al.*, 1999; Garwood *et al.*, 2015). Memory-related functions are regulated by the Insulin in the brain. Brain Insulin signaling pathway exerts a major role in neurons survival along with improving cognitive processes and synaptic plasticity (Beattie *et al.*, 2000; Dou *et al.*, 2005; Huang *et al.*, 2003; Lin *et al.*, 2000; Ma *et al.*, 2003; Mielke *et al.*, 2006; Passafaro *et al.*, 2001; Skeberdis *et al.*, 2001; Wan *et al.*, 1997; Valenciano *et al.*, 2006; Wickelgren, 1998; Zhao *et al.*, 1999). Earlier studies have indicated that Insulin reduces hippocampal inflammation in young rats by decreasing the neuroinflammatory response and enhancing spatial memory in young rats (Adzovic *et al.*, 2015).

Rivastigmine, dual AChE and BuChE inhibitor, is widely used in symptomatic management of AD (Ray *et al.*, 2020). It is known to block hydrolysis of Ach, promotes cholinergic functioning by raising Ach levels and reduces amyloid beta (Aβ) aggregation (Jamshidnejad-Tosaramandani *et al.*, 2021). Also, Rivastigmine has been shown to improve cognitive functions in elder patients without improving peripheral IR. However, brain IR (BIR) is thought to be more serious condition as compared to IR in the periphery. Therefore, BIR and altered cholinergic activity may affect the pathology of AD through various mechanisms (Isik *et al.*, 2012) as impaired Insulin signaling is responsible for the desensitization of the beta-subunit of the Insulin receptor in the brain Insulin signaling pathway rather than in the peripheral signaling pathway. Till now, a clear relationship between IR and AChE inhibition has not been established yet (Isik *et al.*, 2012).

So, Rivastigmine may thus be explored for managing BIR as it may promote neuronal survival by activating the PI3K-Akt signaling pathway (Takada-Takatori *et al.*, 2006) and thus may improve Insulin signaling.

As Insulin and Rivastigmine therapy have been shown to counteract the memory deficit, amyloid deposition, and neuronal degeneration in various studies but till date, no study has been conducted to estimate the effect of Insulin and Rivastigmine treatment in T3D. This study was planned to explore the pharmacological effects of Insulin and Rivastigmine in T3D rats.

MATERIAL AND METHODS

Animals and test conditions

The experimental Wistar rats (200–250 g) were procured from Disease Free Small Animal House (DFSAH), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, and housed in the Animal House of Maharshi Dayanand University (MDU), Rohtak under controlled environmental conditions (relative humidity: 60% ± 10%; temperature: 25°C ± 2°C; alternative light and dark cycle of 12 hours). The rats were placed in the standard polypropylene cages in a group of three rats/cage. The rats had free access to water and food. The study design was approved by the Institutional Animal Ethics Committee (IAEC) of Maharshi Dayanand University (Approval No.: 153-165-17/12/2018).

Chemicals and drugs

STZ (Sigma Aldrich), AlCl$_3$ (Loba Chemie), Insulin (Novo Nordisk), and Rivastigmine (Sun Pharma Ltd.) were used. Drugs were dissolved in saline water before use. All the drugs were injected in a volume of 5 ml/kg through the intraperitoneal (i.p.) route except Insulin which was administered via subcutaneous (s.c.) route.

For the induction of T3D, overnight fasted rats were injected with a single dose of STZ (35 mg/kg, i.p., dissolved in 0.1 M citrate buffer of pH 4.5) followed by the confirmation of hyperglycemia on 3rd day after STZ (Andrade *et al.*, 2018). The rats with a blood glucose level greater than 200 mg/dl were further used (Kumar *et al.*, 2011; Qinna and Badwan, 2015). Thereafter, AlCl$_3$ (12.5 mg/kg., i.p.) was administered for 28 days.

Rats in the vehicle control group received saline. Rivastigmine (1 mg/kg, i.p.) and Insulin (1 IU, sc.) were used as the test drugs (Ismail *et al.*, 2013; John *et al.*, 2015; Liu *et al.*, 2020).

Following 28 days of treatment, the rats have undergone behavioral parameters for determining memory-related functions and neurochemical alterations. The rats with increased insulin, glucose, and amyloid levels in the brain and plasma glucose levels were considered T3D rats.

Evaluation of locomotor activity

Open field test (OFT)

Locomotion in the experimental rats was evaluated by placing each rat in the center region of the open field and observed for 5 minutes. Time spent in the peripheral and central squares and total crossings were noted (Rajasankar *et al.*, 2009).

Several other parameters may also be observed to assess the behavior of rodents such as latency (time to initiate the movement), freezing time (period without any movement), grooming and rearing behavior, and frequency of stretch-attend posture (Hira *et al.*, 2020; Temitayo *et al.*, 2020). Location is generally analyzed by evaluating the time spent in the area of the peripheral and central squares and the total crossing of squares crossed by the rodent (Walsh and Cummins, 1976). The peripheral region is considered a protected area (Hira *et al.*, 2020).

Behavioral assessment of cognitive impairment

EPM test

Elevated plus maze (EPM) comprises two open and two closed/covered arms of dimension 50 × 10 cm each Height of the wall of the closed arm was 40 cm and EPM was elevated at 50 cm from the ground. Closed and open arms were linked through the center square. Each rat was gently placed at the end of one of the open arms. Rat explored the maze for 4 minutes. Transfer latency (TL) (time utilized by a rat to enter one of the covered arms) was recorded. If the rat did not enter any of the closed arms themselves up to 90 seconds, then it was guided to enter into one of the closed/covered arms. The second trial was conducted 24 hours after the first trial and TL was recorded (Itoh *et al.*, 1990; Kulkarni, 2012).

Morris Water Maze (MWM) test

MWM of diameter: 210 cm, height: 51 cm, filled with water up to 35 cm having a temperature of 25 C± 2°C was divided into four quadrants (Q4 was considered as the target quadrant here). Platform (height: 28 cm, width: 10 cm) was kept 2 cm beneath the level of water in Quadrant 4 except in the pre-training trial (Day 1), where the platform was placed at the center of the pool, 1 inch above the water level. Training sessions (Days 2–5) were conducted for 4 consecutive days. Platform position remained the same in Quadrant 4 during the everyday trial. The platform was submerged one inch below the water level. Each rat was made to undergo four successive trials each day. During the trials, each rat was gently placed in the pool facing the wall and the maximum time to swim was allowed up to 60 seconds, the rat was shown a platform for 20 seconds. The rat was immediately removed from the pool when it located the platform before 60 seconds. If the rat was not able to locate the platform after 60 seconds, it was guided to locate the platform and placed for additional 20 seconds on the platform. Each test was performed roughly at the same time to reduce the variability in the performance of rats. After 24 hours of the last day of the training session, a probe test was performed on the 6th day to estimate spatial reference memory. In the probe trial, after the removal of the platform from the pool, the rat was allowed to swim for 240 seconds. Time spent in Quadrant 4 indicated memory of rats (Morris, 1984).

Biochemical estimation

Blood and brain sample collection

After the behavioral parameters, rats were sacrificed; blood was collected and centrifuged at 2,500 × g for 15 minutes.

Whole brain of each rat was carefully isolated. The brains were homogenized in an ice bath after adding 10 volumes of 0.1 M phosphate buffer (pH 7.4). The homogenate was then centrifuged for about 15 minutes at 10,000 × g. The resultant supernatant liquid was used for biochemical assessment.

Aβ estimation

In the Congo-Red (CR) fluorescence method, a fresh solution of CR was passed through a filter (0.2 µm) in cold potassium phosphate buffer (5 mm) containing sodium chloride (NaCl) (150 mM) maintained at a pH of 7.4. This CR solution was then added to 100 µg/ml protein solution, finally resulting in a 5 µM concentration and the samples were mixed for 15 seconds. The absorption spectrum was calculated and noted in the range of 400–700 nm in UV–Vis spectrophotometer (Shimadzu-1800). The spectrum of CR solution with protein was contrasted with alone CR solution. An increase in absorption and red shift of the absorption spectrum toward 540 nm indicated the production of amyloid formation (Meehan *et al.*, 2004).

Insulin estimation

Insulin level in the brain was measured using a Beckman Coulter kit based on the chemiluminescent assay method.

Glucose estimation

The glucose level in the brain was measured using the Glucose estimation kit (ERBA) based on the GOD-POD method. The blood glucose levels were estimated using a glucometer (Accucheck).

Brain malondialdehyde (MDA) assay

MDA level was assessed using the UV-Vis spectrophotometer (Shimadzu-1800). Absorbance was noted at 532 nm (Wills, 1966).

Brain nitrite estimation

Brain nitrite estimation was carried out by the method described by Green *et al.* (1982). The supernatant was mixed with Griess reagent and the mixture was incubated for 10 minutes at 37°C. The absorbance was noted with UV–Vis spectrophotometer (Shimadzu-1,800) at 546 nm.

Experimental groups

Experimental groups ($n = 8$):
Group-1. Control group: The rat in this group received saline;
Group-2. T3D rats: STZ (35 mg/kg, i.p.) + (After 3 days, $AlCl_3$ 12.5 mg/kg., i.p. daily for 28 days).
Group-3. Insulin-treated T3D rats: STZ (35 mg/kg, i.p.) + Insulin (IU s.c. daily for 28 days) 30 min before the $AlCl_3$ administration)
Group-4. Rivastigmine-treated T3D rats: STZ (35 mg/kg, i.p.) + Rivastigmine (1 mg/kg, i.p. daily for 28 days) 30 min before the $AlCl_3$ administration)

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test using the software GraphPad Prism version 9. Data expressed as mean ± SEM. $p < 0.05$ were considered as statistically significant.

RESULTS

Insulin and Rivastigmine effect on locomotion in T3D rats

Time spent in the center square ($F_{3,28} = 1.896$, $p = 0.1533$) (Fig. 1A), peripheral square ($F_{3,28} = 1.220$, $p = 0.3207$) (Fig. 1B),
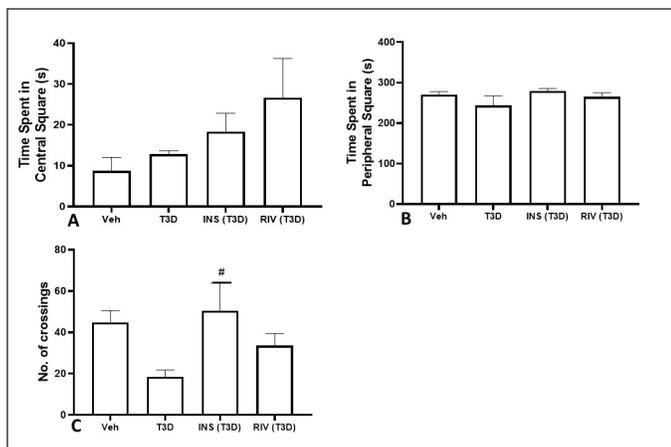
Figure 1. Effect of various treatments on locomotion in OFT test. Data are expressed as mean ± SEM (*n* = 8). #*p* < 0.05 versus type-3 diabetes group.

Figure 2. Effect of insulin and Rivastigmine on the performance of rats in EPM test. Data are expressed as Mean ± SEM (*n* = 8). #*p* < 0.05, ##*p* < 0.01 versus type-3 diabetes group, *******p* < 0.01 versus vehicle treated group.

and total crossings (F$_{3,28}$ = 3.043, *p* = 0.0452) (Fig. 1C) after Insulin and Rivastigmine administration were determined using one-way ANOVA. Tukey's *post hoc* analysis suggested no effect of Rivastigmine on the time spent in the center and periphery square and total crossings in comparison to their respective control. However, the Insulin treatment increased the frequency of crossings significantly as compared to its control (*p* < 0.05).

Insulin and Rivastigmine effect on the memory-related alterations in T3D rats in EPM test

The effect of Insulin and Rivastigmine treatment on TL on day 1 (F$_{3,28}$ = 3.433, *p* < 0.0304) (Fig. 2A) and day 2 (F$_{3,28}$ = 6.448, *p* = 0.0019) (Fig. 2B) in rats was determined. Tukey's *post hoc* analysis suggested significant reduction in TL by the Insulin (*p* < 0.01) and Rivastigmine (*p* < 0.05) treatment in comparison to their control.

Insulin and Rivastigmine effect on the memory-related impairment in T3D rats in MWM test

The results suggested that T3D rats spent less time in the target quadrant resulting in impaired cognition. One-way ANOVA indicated the effect of Insulin and Rivastigmine on time in Q4 (F$_{3,28}$ = 2.869, *p* = 0.542) (Fig. 3A); frequency of platform crossing (F$_{3,28}$ = 3.039, *p* = 0.0454) (Fig. 3B) and frequency of target quadrant crossing (F$_{3,28}$ = 0.4806, *p* < 0.6984) (Fig. 3C). Tukey's *post hoc* analysis suggested no effect of Insulin and Rivastigmine on the time spent in Q4, number of center crossings, platform crossings, and crossing across Q4 in comparison to their respective controls. However, Insulin treatment reduced the frequency of platform crossings significantly in comparison to its control (*p* < 0.05).

Insulin and Rivastigmine effect on blood and brain glucose levels of T3D rats

The results obtained has indicated the effect of Rivastigmine and Insulin administration on glucose level in the blood (F$_{3,28}$ = 38.12, *p* < 0.001) (Fig. 4A) and brain (F$_{3,28}$ = 128, *p* = 0.001) (Fig. 4B). Tukey's *test* has suggested a significant rise in glucose levels in the blood and brain of T3D rats in comparison to the control (*p* < 0.001). However, the Insulin and Rivastigmine treatment decreased the blood glucose levels significantly in comparison to its control (*p* < 0.001).
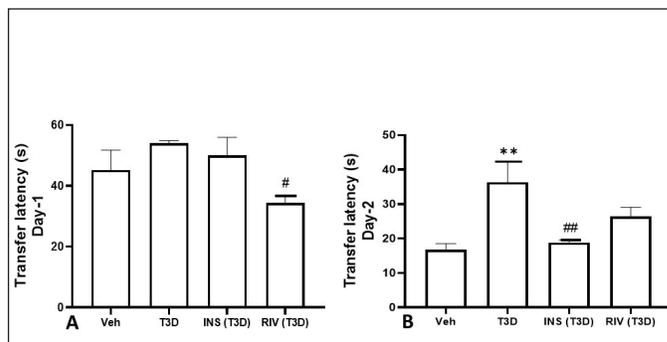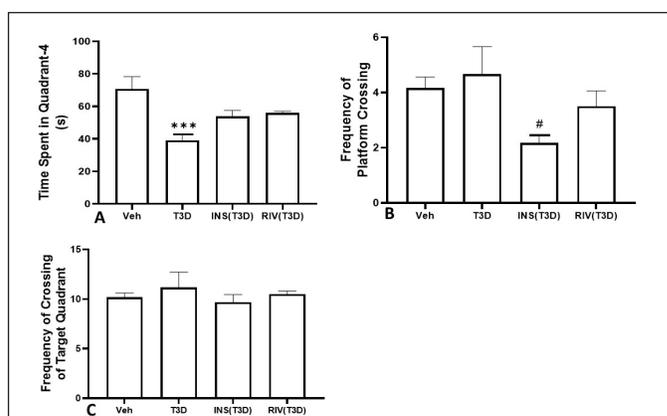
Figure 3. Effect of insulin and Rivastigmine on the performance of rats in MWM test. Data are expressed as mean ± SEM (*n* = 8). #*p* < 0.05 versus type-3 diabetes group, *******p* < 0.001 versus vehicle treated group.

Insulin and Rivastigmine effect on brain Insulin level of T3D rats

Administration of Rivastigmine has revealed reduced Insulin levels in the brain (F$_{3,28}$ = 7.858, *p* = 0.0006) (Fig. 5). Tukey's test suggested a significant rise in Insulin levels in the brain of T3D rats as compared to its control (*p* < 0.01). However, Rivastigmine treatment decreased the brain Insulin levels significantly in comparison to its control (*p* < 0.01).

Insulin and Rivastigmine effect on the brain Aβ level in T3D rats

Insulin and Rivastigmine administration to T3D rats reduced the brain Aβ levels (F$_{3,28}$ = 431.3, *p* < 0.001) (Fig. 6). Tukey's test has suggested a significant rise in Aβ level in the brain in comparison to the control (*p* < 0.001). However, Rivastigmine and Insulin treatment reduced the brain Aβ levels significantly in comparison to its control (*p* < 0.001).

Insulin and Rivastigmine effect on the nitrite and MDA level in the brain of T3D rats

One-way ANOVA analysis of the data has indicated the effect of Rivastigmine and Insulin treatment on brain nitrite levels (F$_{3,28}$ = 0.1880, *p* = 0.9037) (Fig. 7) and MDA level in the brain (F$_{3,28}$ = 3.895, *p* ≤ 0.0192) (Fig. 8). Tukey's test suggested
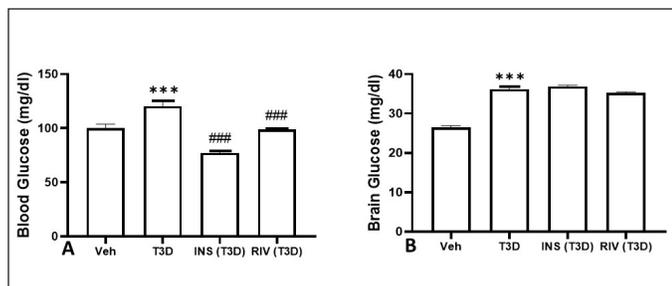
Figure 4. Effect of insulin and Rivastigmine on the glucose level of rats. Data are expressed as mean ± SEM (*n* = 8). ###*p* < 0.001 versus type III diabetes group,***p* < 0.001 versus vehicle treated group.
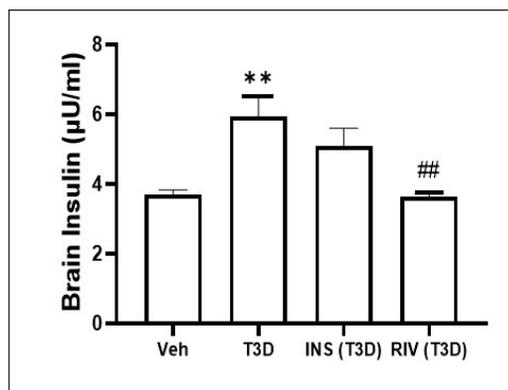
Figure 5. Effect of insulin and Rivastigmine on the insulin level of rats. Data are expressed as mean ± SEM (*n* = 8). ##*p* < 0.01 versus type III diabetes group, ****p* < 0.01 versus vehicle treated group.
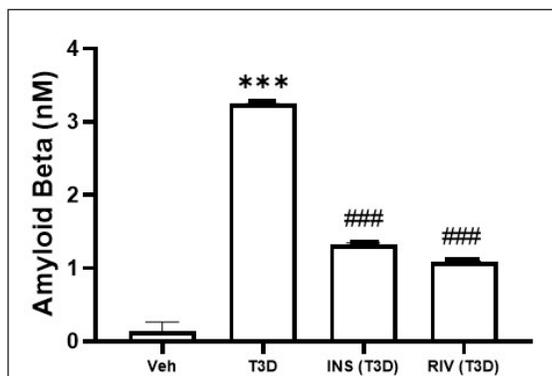
Figure 6. Effect of insulin and Rivastigmine on the Aβ level of rats. Data are expressed as mean ± SEM (*n* = 8). ###*p* < 0.001 versus type III diabetes group, ***p* < 0.001 versus vehicle treated group.

non-significant effect on MDA and nitrite levels in the brain in comparison to their control.

DISCUSSION

In this study, the combination of STZ (35 mg/kg, i.p., single-dose administration) and AlCl$_3$ [12.5 mg/kg daily (i.p.) × 28 days] was used to induce T3D in experimental rats. It was
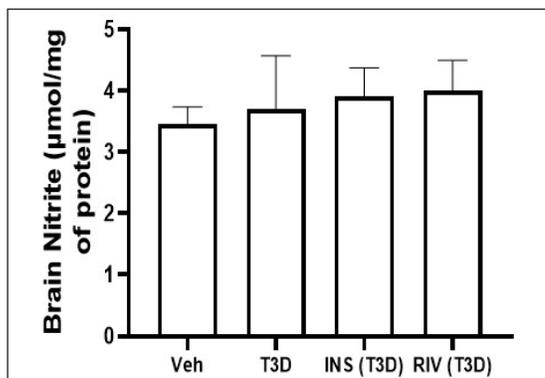
Figure 7. Effect of insulin and Rivastigmine on brain nitrite level of rats. Data are expressed as mean ± SEM (*n* = 8).
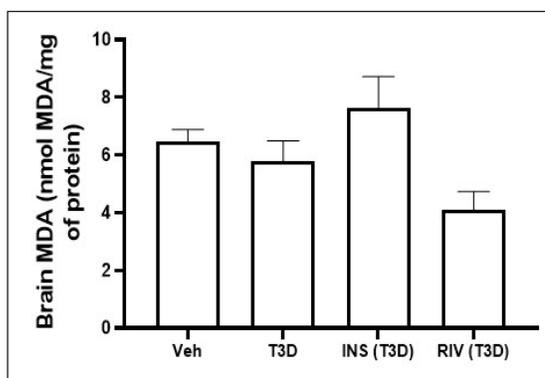
Figure 8. Effect of insulin and Rivastigmine on brain MDA level of rats. Data are expressed as mean ± SEM (*n* = 8).

observed that AlCl$_3$ and STZ administration in rats has caused behavioral changes in EPM and MWM tests. Further, AlCl$_3$ and a single dose of STZ administration in rats have raised the brain glucose, Insulin, and Aβ levels significantly in comparison to the control. Thus, the present experimental study revealed that intraperitoneal administration of STZ (35 mg/kg, once) and AlCl$_3$ (12.5 mg/kg × 28 days) in rats is responsible for the development of hyperglycemia, hyperinsulinemia, and an increase in Aβ level.

The effect of Insulin and Rivastigmine treatment on the various behavioral and neurochemical parameters of T3D rats was estimated which revealed that Rivastigmine has no significant effect on time spent at the center and periphery and the number of square crossings of T3D rats in the OFT. In contrast to this study, Rivastigmine has shown improvement in locomotor and exploratory behavior of aluminum-treated rats in the OFT but did not significantly alter latency to initiate movement suggesting no effect on anxiety in these rats (Abdel-Aal *et al.*, 2011). This effect may be due to the peripheral cholinesterase inhibitory action of Rivastigmine (Wilkinson *et al.*, 2004). However, Insulin administration has increased the number of square crossings indicating exploratory behavior of animals.

In EPM test, Rivastigmine did not affect the TL of day 2 of T3D rats while Insulin decreased the TL of day 2 of T3D rats suggesting the better activity of Insulin. In another similar study, Insulin administration has shown a significant reduction in

TL in EPM in Aluminum treated rats (Alam and Bansal, 2020). Peripheral Insulin administration has been shown to improve memory functions in animal models of diabetes (Zhao *et al.*, 2004). In contrast to our study, cognitive decline induced in aluminum-treated rats was reduced by Rivastigmine administration, as it improved the cognitive deficits observed in various behavioral tests. However, this effect was dose-dependent. A low dose of Rivastigmine (0.5 mg/kg) did not exert any significant effect on the rats' performance in behavioral tests whereas a high dose of Rivastigmine (2.5 mg/kg) produced its maximum effect (Abdel-Aal *et al.*, 2011). This dose-dependent effect was mediated based on the extent of cholinesterase inhibition. Bejar *et al.* (1999), suggested that Rivastigmine at a dose of 0.5, 0.75, and 2.5 mg/kg produced 21%, 40%, and 62% reduction in cholinesterase activity, respectively (Táyebati *et al.*, 2004). Rivastigmine acts as a dual reversible inhibitor of both AChE and BuChE (Muller, 2007; Táyebati *et al.*, 2004) and has been shown to lower the glycated hemoglobin in diabetic patients (Wills, 2009). It has been reported that Rivastigmine (5 mg/kg) improved the memory and learning deficits caused by $AlCl_3$ (Thippeswamy *et al.*, 2013).

T3D rats have shown a reduction in the time spent in the target quadrant in the MWM test indicating memory impairment. Insulin and Rivastigmine had no effect on the time spent and frequency of target quadrant (Q4) crossing by T3D rats. However, Insulin decreased the frequency of platform crossing by T3D rats. In a previous study, Insulin administration has also shown a significant reduction in escape latency in MWM in Aluminum treated rats (Alam and Bansal, 2020). Further, 6 weeks of intra-nasal Insulin treatment has shown improvement in the spatial memory impairment in MWM in the rats which were administered STZ via intracerebroventricular (icv) route. Thus, confirming the positive effect of Insulin on cognition (Guo *et al.*, 2017).

Administration of Rivastigmine reduced the brain Insulin level of T3D rats suggesting that Rivastigmine may be responsible to regulate brain Insulin signaling. However, no effect of Insulin administration was observed on brain Insulin levels. These findings are in support of previously conducted studies, where there was no difference was observed in the brain Insulin levels between the intraperitoneally administered Insulin (IU/ml) and STZ (3 mg/kg, icv) groups. However, a dose-dependent increase in peripheral Insulin levels was seen in the intraperitoneally administered Insulin group (Lv *et al.*, 2020). In this study, no significant effect was observed in oxidative stress markers such as nitrite and MDA levels whereas previous studies have shown that Rivastigmine (2.5 mg/kg, p.o.) reduced the elevated nitrite level significantly (Khan *et al.*, 2013), diminished the MDA level (Chen *et al.*, 2021; John *et al.*, 2015). The observed beneficial effect may be dose-dependent.

Insulin stimulation promotes the extracellular excretion of Aβ and reduces its intracellular aggregation (Gasparini *et al.*, 2001) whereas altered Insulin signaling affects amyloid precursor protein processing, Aβ clearance, leading to raised neurotoxic effects of Aβ (Delikkaya *et al.*, 2019). Aβ exerts an effect on Insulin signaling, either by reducing its affinity or by competing with and inhibiting the binding of Insulin to the Insulin receptor (Xie *et al.*, 2022). In this study, Insulin and Rivastigmine have reduced the level of amyloid deposits in the brain. These findings are in support of previously conducted studies, where Rivastigmine (0.5 mg/kg) has

significantly reduced the plasma Aβ levels of $AlCl_3$ treated rats (Dulla and Bindhu, 2022). Thus, the reduction of amyloid burden improves learning and memory and may halt the process of neurodegeneration. Rivastigmine reduces Aβ brain load by promoting its clearance across the BBB by upregulating Lipoprotein receptor-related protein 1 and P-glycoprotein (Mohamed *et al.*, 2016). Rivastigmine may also alter α-secretase levels and shift amyloid precursor protein processing toward non-amyloidogenic pathway and may modify the disease progression pathway (Ray *et al.*, 2020).

Findings from this study indicate that Rivastigmine might have exerted a beneficial effect by compensating the cholinergic deficits either by increasing the choline acetyltransferase levels or by inhibiting the breakdown of acetylcholine (Táyebati *et al.*, 2004). Rivastigmine has also reduced brain Insulin levels, so, it might have exerted an effect on the Insulin signaling pathway and reduced hyperinsulinemia. Also, Insulin might have exerted a beneficial effect by activating Insulin signaling pathways involving NMDA receptors, PI3K and Akt/PKB activity, and GSK3β inactivation (Neumann *et al.*, 2008).

CONCLUSION

This study has demonstrated that both Insulin and Rivastigmine treatments have reduced brain amyloid levels while Rivastigmine treatment alone has reduced the brain Insulin levels. Further, Insulin treatment in T3D rats has improved memory in the EPM test. In conclusion, the obtained results hold the potential to offer a suitable combination of Insulin and Rivastigmine treatment to control both behavioral and biochemical disturbances in the management of T3D.

AUTHOR CONTRIBUTIONS

Ms. Abhilasha Ahlawat: Collection of data and write the manuscript.
Dr. Vaibhav Walia: Statistical analysis.
Prof. Munish Garg: Study Design and Proof Reading.

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CONFLICTS OF INTEREST

The authors declared no conflicts of interest.

ETHICAL APPROVALS

The experimental study design was approved by Institutional Animal Ethics Committee of Maharshi Dayanand University (Approval no.: 153-165-17/12/2018).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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