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Research Article

Modulation of Metabolic and Cognitive Parameters by Withania somnifera Root Extract in High-Fat Diet and Streptozotocin (HFD-STZ) Induced Type 2 Diabetic Rats

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Abstract

Objective: To study the effects of *Withania somnifera* root extract on metabolic and cognitive parameters in rats with induced type 2 diabetes mellitus.

Methods: SD rats were fed with High-fat diet (HFD) for 8 weeks followed by intraperitoneal administration of low dose Streptozotocin (STZ; 35 mg/kg) to induce type 2 diabetes mellitus (T2DM). Fasting blood glucose, serum triglycerides and serum cholesterol levels were estimated after four weeks of STZ administration. After the confirmation of diabetes, diabetic animals were treated with *Withania somnifera* extract (WS) and Pioglitazone (PZ) for another 8 weeks. At the end of the treatment, cognitive analysis was done by Morris water maze test (MWM). Thereafter, animals were sacrificed and blood samples were taken for biochemical analysis and insulin resistance was calculated.

Results: Fasting blood glucose, triglyceride, cholesterol was found to be significantly elevated in HFD-STZ induced diabetic rats as compared to control animals. Treatment with WS and PZ for 8 weeks significantly lowered these levels. In MWM test, diabetic animals showed weaker learning and retention as indicated by increased escape latency time (ELT) and lesser time spent in target quadrant (TSTQ) respectively, as compared to control animals. However, treatment with WS for 8 weeks significantly reduced ELT and increased TSTQ as compared untreated diabetic rats. WS treatment also significantly improved serum adiponectin, and brain insulin levels as compared to diabetic rats.

Conclusion: The data suggests that treatment with root extract of *Withania somnifera* and Pioglitazone lowered the metabolic parameters and insulin resistance in type 2 diabetic animals when compared with normal control group. Also, it was found that T2DM adversely affects memory and treatment with these drugs improved learning and retention in diabetic animals. Thus, root extract of *Withania Somnifera* may have therapeutic effects for the management of cognitive deficit due to type 2 diabetes mellitus.

Keywords: *Withania somnifera; Streptozotocin (HFD-STZ); Type 2 Diabetic Rats*

Introduction

Diabetes mellitus (DM) is a chronic and most common metabolic disorder and is a gradually rising major health concern worldwide. Hyperglycemia is the main feature of DM that is caused due to insufficient secretion or action of Insulin. DM is categorized into type 1 (T1DM) and type 2 (T2DM). T1DM caused due to insufficient secretion of insulin by pancreatic β-cells whereas T2DM is caused due to defective insulin secretion by pancreatic β-cells as well as decrease in the response of insulin-responsive cells to the bioavailable insulin i.e. insulin resistance (IR). T2DM is the much more prevalent form of diabetes and approximately 90% of diabetes mellitus cases are T2DM. T2DM is generally associated with sedentary lifestyle, intake of high calorie diet, obesity and aging [1]. Less physical activity, frequent consumption of foods rich in saturated fats and sugar cause insulin resistance and obesity leading to a variety of metabolic disorders like T2DM. As a result of insulin resistance (IR), β-cell compensatory mechanism gets activated in the early stage of the disease which causes hyperinsulinemia i.e. elevated insulin levels in blood. As the disease progresses, there is impairment in the insulin secretion due to pancreatic β-cells dysfunction, which results in hyperglycemia [2]. T2DM is the major risk factor for cardiovascular diseases such as myocardial infarction, stroke, atherosclerosis etc. Chronic hyperglycemia and dyslipidemia are the major hallmarks of the disease that may contribute to the development of macrovascular and microvascular complications [3].

Insulin is an anabolic peptide hormone secreted by pancreatic β cells. Insulin has various actions in the body but regulation of glucose metabolism is its primary function. Insulin receptors are differentially distributed in various body tissues and exhibit a multiorgan signal transduction pathway by tyrosine kinase activity. Insulin receptors on myocytes and adipocytes increase glucose uptake by increasing the translocation of glucose transporters (mainly GLUT4) to the cell surface, so that glucose can be stored for energetic situations [4]. β cell dysfunction causes interruption in the release of insulin that results in the development of type 1 and type 2 diabetes mellitus. Insulin resistance is the main hallmark of type 2 diabetes mellitus (T2DM) which is characterised by insulin insensitivity and deficiency in its use by peripheral tissues. This results in increase in the insulin demand and β cell compensatory mechanism which causes increase in β cell mass and in insulin secretion in the blood, known as hyperinsulinemia. Persistent hyperinsulinemia initiates variety of mechanisms like decrease in insulin receptor expression, inhibition of insulin receptor kinase activity by insulin receptor substrate ½ (IRS 1/2) tyrosine phosphorylation that leads to peripheral insulin resistance [5].

Insulin enters brain via receptor mediated active transport through blood brain barrier (BBB). It is an adenosine triphosphate (ATP) dependent process. This transport demonstrates saturable kinetics and therefore the transport decreases with increase in blood insulin level [6]. AD patients exhibit impaired insulin signaling that is similar to that observed in T2DM. In brain, insulin plays a pivotal role in the neuronal function by regulating energy metabolism, growth survival and differentiation via insulin signaling and plays a key role in learning and memory [7]. Although brain is not the target tissue for insulin but insulin signalling has been implicated in various brain functions like cognition. It has been observed that people with insulin resistance are at increased risk for dementia. Also, in rodents it has been observed that insulin receptors are present in various brain regions like hippocampus and cortex and insulin effects on brain is interrupted by feeding them with high-fat diet [8]. According to recent studies, insulin along with other growth factors like brain derived neurotrophic factor (BDNF) transmits the signals to regulate cognitive functions mediated by hippocampus. Insulin resistance caused by diabetes down-regulates the signaling pathways of insulin in hippocampus and therefore leads to impairment in memory and synaptic plasticity [9].

T2DM and AD both are prevalent with aging. Individuals with type 2 diabetes mellitus, obesity and other metabolic syndromes are at high risk to develop AD that may be due to mitochondrial dysfunction [10]. T2DM leads to structural as well as physiological change that causes cognitive deficits and the condition is known as diabetic encephalopathy [11]. Persistent high blood glucose levels in diabetics damages small blood vessels which leads to microvascular complications like diabetic neuropathy, nephropathy and retinopathy whereas damage to large blood vessels leads to macrovascular complications like cerebrovascular disease, cardiovascular disease and stroke [12].

Over a course of time, high blood sugar due to insufficient action of insulin (Insulin resistance) starts affecting brain cognitive functions which lead to mild cognitive impairment (MCI) in early stage and AD in later stage hence, AD has been considered as brain type diabetes [13].

Currently various medications are available for the management of T2DM like Thiazolidinediones, metformin, α-glucosidase inhibitors etc but there is no drug therapy that provides definite solution for prevention or management of cognitive deficit induced by T2DM. In traditional system of medicine, the roots of *Withania somnifera* plant are categorised as rasayanas (Rasa: Juice, Ayana: Path), which literally means the path of juice [14]. The consumption of specific rasayanas in a recommended pattern is supposed to increase the rasa in the cells which rejuvenates the system [15]. Hence, the present study was designed to investigate the effects of root extract of *Withania Somnifera* extract on experimental model of type 2 diabetes mellitus induced cognitive deficit and the possible mechanisms mediating these effects.

Materials and Methods

Subjects

Sprague Dawley (SD) rats (200 - 250g) were used for the study and each experimental group was comprised of 5 animals. The rats were housed under standard laboratory conditions (22 ± 2°C, 12 hour light/dark cycle - lights on at 0800 hours). Care of animals has been taken as per guidelines of CPCSEA for use of animals in Scientific Research with approval of Institutional Animals Ethics Committee (IAEC).

High fat diet- streptozotocin (HFD-STZ) Alzheimer model

After one week of acclimatization period, Sprague Dawley (SD) rats (220 - 280g), were divided into two groups. Group I: Twelve animals (n = 12) served as normal control group and given normal pellet diet for 8 weeks and Group II: (30 animals) were fed with in-house prepared high fat diet (HFD) (60% calories as fat) the diet composition includes powdered (Normal Pellet Diet) NPD (425 g/kg), lard (310 g/kg), casein (250 g/kg), cholesterol (10 g/kg), DL-methionine (3 g/kg), yeast powder (1 g/kg) and sodium chloride (1 g/kg) for a period of 8 weeks. After 8 weeks of dietary manipulation animals were kept on overnight fasting, Group II animals then injected intraperitoneally (i.p) with a low dose of streptozotocin (STZ) 35 mg/kg dissolved in 0.1 M citrate buffer pH 4.5, while the respective normal control rats were treated with vehicle citrate buffer in a dose volume of 1 ml/kg, i.p. [16,17]. The rats were allowed to continue to feed on their respective diets until the end of the study. The STZ induced hypoglycemic mortality was avoided by feeding the Group II animals with 20% glucose solution for 24 hrs [18].

After 4 weeks of STZ administration, the fasting blood glucose level was determined from the tail vein by glucometer. Rats with fasting glucose level ≥ 200 mg/dL were considered as diabetic and selected for further pharmacological screening. Blood (1.0 ml) was collected from retroorbital plexus of the rats under mild anesthesia using capillary into eppendorff tubes. The serum was be separated by centrifugation and used to measure the levels of triglyceride and cholesterol by semi-automatic analyzer. After 4 weeks of STZ administration, body weight and fasting blood glucose levels of all the animals were measured and control animals were divided into two groups as described below:

- Normal control (n = 6): Normal control animals were treated with 1% Na-CMC suspension orally for 8 weeks of treatment period.
- Normal control treated with *Withania somnifera* at the dose of 300 mg/kg, p.o (NC+WS-300; n = 6): Control animals in this group were treated with root extract of *Withania Somnifera* 300 mg/kg/day orally for 8 weeks.

Diabetic animals were further divided into four groups containing 6 animals (n = 6) each and respective drugs were administered (as shown below):

- Diabetic control: Animals were orally administered with Na-CMC suspension for 8 weeks.
- WS-100: Animals were treated with the root extract of *Withania somnifera* 100 mg/kg/day, orally for eight weeks.
- WS-300: Animals were orally administered with root extract of *Withania somnifera* 300 mg/kg/day.
- PZ-20: Animals were treated with Pioglitazone 20 mg/kg/day, orally for 8 weeks.

After 8 weeks of treatment with standard (Pioglitazone) and test (*Withania somnifera*) drugs, body weight, fasting blood glucose levels were assessed. The neurobehavioural parameters were assessed by morris water maze test. After the screening for neurobehavioural parameters, animals were sacrificed and blood samples were collected by cardiac puncture and serum was isolated by centrifugation. Brain of the animals were removed and pre-frontal cortex and hippocampus were isolated from whole brain. Insulin levels of serum, prefrontal cortex and hippocampus was evaluated by ELISA according to the manufacturer's protocol. Also, serum triglycerides and cholesterol levels were estimated.

Parameters assessed

After 8 weeks of treatment with test and standard drugs the animals were screened with neurobehavioral and biochemical parameters.

Body weight, fasting blood sugar, serum triglyceride and serum cholesterol levels

All the four parameters of each animal were recorded at the end of 12th week i.e. after four weeks of STZ administration (before starting drug treatment). At the end of 20 weeks, all the animals were kept on 12 hours fasting and their body weights and fasting blood sugar levels were assessed by tail vein using glucometer.

Neurobehavioral studies

After 12 hrs of OGTT, neurobehavioural parameters were assessed by Morris water maze test.

Morris water maze test (MWM)

Animals were subjected to the MWM to analyze cognitive function at the end of treatment with drugs. In brief, a circular tank (160 cm diameter and 50 cm height) was filled with warm water (22 - 24°C) to a height of 30 cm. The pool area was divided into four quadrants (NE, SE, SW and NW) by four points (E, S, W and N), equally spaced along the circumference of the pool. A circular escape platform (12 cm diameter and 28 cm height) was fixed in the middle of quadrant NE, 2 cm below the surface of water for the non-visible trial. All the rats received the four non-visible platform trials (one from each starting point) everyday for the first 4 days and a probe trial on the 5th day by removing the platform. In the non-visible platform trials, if the animal was unable to reach the platform in the given time interval (120s), it was guided gently to the platform and allow to remain there for 30 seconds. The time taken by the animal to reach the platform (escape latency time) was recorded on day 1 - 4. On day 5, the time spent in target quadrant (TSTQ) in search of the missing platform was recorded using stopwatch and was used for further statistical analysis. TSTQ is an indicator of memory retention [19,20].

Serum triglyceride, cholesterol, insulin and adiponectin levels

After neurobehavioral studies, the animals were sacrificed by ketamine overdose and blood samples were taken by cardiac puncture and serum was separated. Serum insulin level was determined by ELISA and triglyceride and cholesterol levels were determined by using commercial kits according to the manufacturer's protocol.

Brain insulin levels

Insulin levels of hippocampus and prefrontal cortex were determined by ELISA according to manufacturer's protocol.

Statistical analysis

The data is expressed as Mean ± SEM. Comparisons among different groups is analyzed by one way ANOVA followed by Tuckey's post hoc analysis. Non-parametric statistical tests (Mann-Whitney U test) is used for analyzing data wherever appropriate. A 'P' value of less than 0.05 is considered as the level of significance for all statistical tests.

Results

Effect of *Withania somnifera* (WS) on serum fasting blood glucose levels in diabetic rats

Figure 1A and 1B shows serum fasting blood glucose levels after 12 and 20 weeks of the study respectively. A significant increase in blood glucose level ($p < 0.001$) was observed after 4 weeks of high-fat diet and streptozotocin (HFD-STZ) treatment as compared to normal control animals (Figure 1A). After 8 weeks treatment with *Withania somnifera* (WS) at the dose of 100 mg/kg and 300 mg/kg, the fasting blood glucose levels were found to be significantly reduced $p < 0.05$, $p < 0.01$, respectively. Treatment of Normal control group with WS did not induce any significant change.

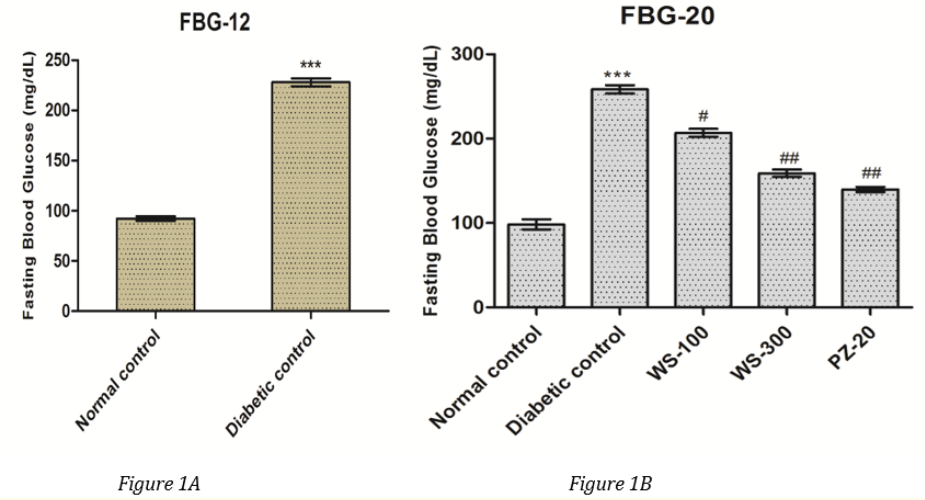

Figure 1A *Figure 1B*

Figure 1: *Fasting blood glucose (FBG) levels after 12 and 20 weeks (Figure 1A and 1B respectively) of the study. Values are expressed as Mean ± SEM; ***p < 0.001 when compared with control group. #p < 0.05, ##p < 0.01 when compared with diabetic control group.*

Effect of *Withania somnifera* (WS) on serum triglycerides levels in diabetic rats

Figure 2A and 2B shows serum triglycerides levels after 12 and 20 weeks of the study respectively. After 4 weeks of HFD-STZ treatment, serum triglycerides level of diabetic animals was found to be significantly increased ($p < 0.01$) as compared to normal control group, as shown in figure 2A. Treatment with WS at the dose of 100 mg/kg did not significantly reduce the TG level. At the dose of 300 mg/kg, WS reduced TG level significantly ($p < 0.05$) as compared to diabetic control group (Figure 2B). Treatment of normal control group with WS did not induce any significant change in TG level.

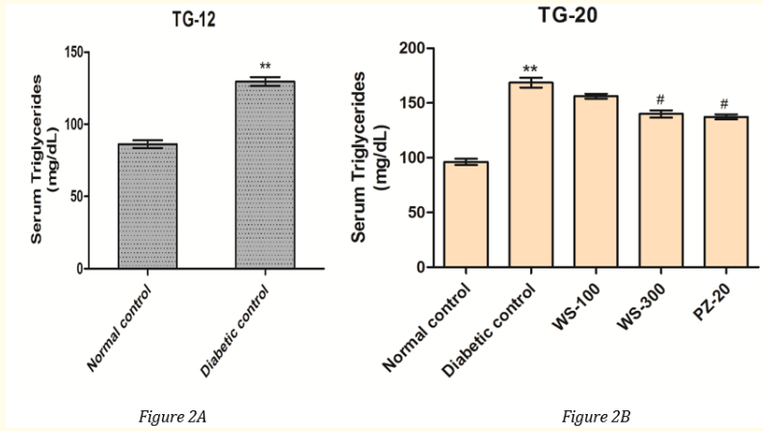

Figure 2A *Figure 2B*

Figure 2: *Serum triglyceride (TG) levels after 12 and 20 weeks (Figure 2A and 2B respectively) of the study. Values are expressed as Mean ± SEM; **p < 0.01 when compared with control group. #p < 0.05, when compared with diabetic control group.*

Effect of *Withania somnifera* (WS) on serum total cholesterol levels in diabetic rats

Figure 3A and 3B shows serum cholesterol levels after 12 and 20 weeks of the study respectively. After 4 weeks of HFD-STZ treatment, serum triglycerides level of diabetic animals was found to be significantly increased ($p < 0.01$) as compared to normal control group, as shown in figure 2A. Treatment with WS at the dose of 100 mg/kg did not significantly reduce the TG level. At the dose of 300 mg/kg, WS reduced TG level significantly ($p < 0.05$) as compared to diabetic control group (Figure 2B). Treatment of normal control group with WS did not induce any significant change in TG level.

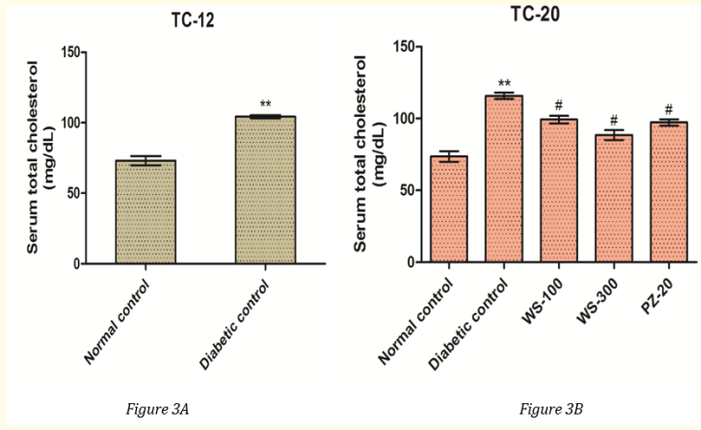

Figure 3A *Figure 3B*

Figure 3: *Serum total cholesterol (TC) levels after 12 and 20 weeks of the study. Values are expressed as Mean ± SEM; **p < 0.01, when compared with control group. #p < 0.05, when compared with diabetic control group.*

Effect of *Withania somnifera* on brain insulin levels in diabetic rats

Figure 4A and 4B shows insulin levels in prefrontal cortex and hippocampus after 20 weeks of the study respectively. After 8 weeks of the induction of T2DM, insulin level of prefrontal cortex and hippocampus in diabetic animals was found to be significantly reduced ($p < 0.001$) as compared to normal control group. Treatment with WS at the dose of 100 mg/kg for 8 weeks did not significantly increase the brain insulin level. At the dose of 300 mg/kg, WS significantly increased insulin level ($p < 0.05$) as compared to diabetic control group as shown in figure 4A and 4B. Treatment of normal control group with WS did not induce any significant change in brain insulin level.

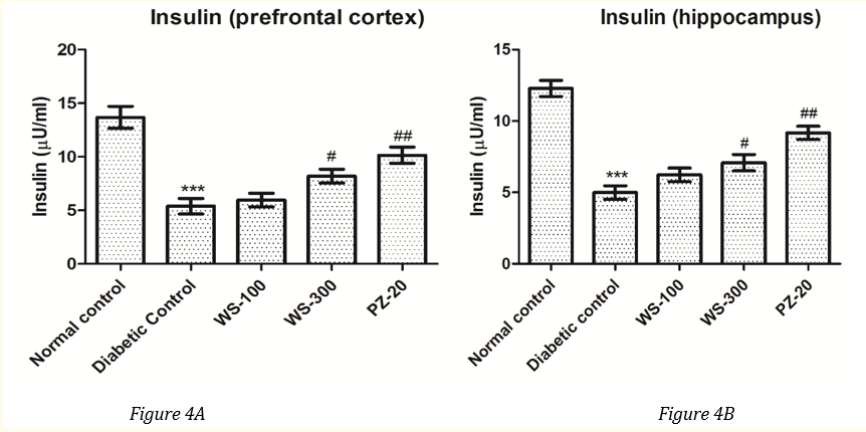

Figure 4A *Figure 4B*

Figure 4: *Brain Insulin levels in hippocampus and prefrontal cortex after 20 weeks of the study. Values are expressed as Mean ± SEM; ***p < 0.001, when compared with control group. #p < 0.05, ##p < 0.01 when compared with diabetic control group.*

Effect of *Withania somnifera* on the serum markers of Insulin resistance (fasting blood glucose, insulin) and adiponectin

At the end of the treatment (after 20 weeks), fasting blood glucose level, serum insulin and serum adiponectin levels were measured. It was found that, diabetic animals showed significant increase in fasting blood glucose level ($p < 0.001$) as compared to normal control group. Treatment with WS for 8 weeks at the dose of 100 and 300 mg/kg significantly restored the glucose level ($p < 0.05$, $p < 0.01$ respectively) as shown in table 1 and figure 1B.

Serum insulin level of the animals of diabetic group was found to be significantly increased ($p < 0.01$) as compared to normal control group. Treatment of diabetic animals with WS-300 significantly lowered the insulin level ($p < 0.05$) as compared to diabetic control animals (Table 1) however treatment with WS-100 did not show any significant difference.

Insulin resistance was calculated by homeostatic model assessment of insulin resistance (HOMA-IR) and it was found that induction of diabetes significantly increased insulin resistance ($p < 0.01$) in diabetic control animals as compared to normal control group. 8 weeks treatment with WS-100 and WS-300 significantly lowered the insulin resistance ($p < 0.05$ and $p < 0.01$, respectively) as compared to diabetic animals (Table 1).

Serum adiponectin level of untreated diabetic animals was found to be decreased significantly ($p < 0.001$), compared with normal control animals. Treatment with WS-300 increased serum adiponectin level significantly ($p < 0.01$) as compared to the animals of diabetic control group (Table 1).

Group	Fasting blood glucose (mg/dl)	Serum insulin (µU/ml)	HOMA-IR	Serum Adiponectin (ng/ml)
Normal control	98.33 ± 5.93	17.76 ± 1.52	4.32 ± 0.50	116.90 ± 5.33
NC+WS-300	103.60 ± 2.42	16.56 ± 1.85	4.21 ± 0.44	114.5 ± 4.58
Diabetic Control	258.30 ± 4.99***	29.79 ± 1.77**	18.75 ± 1.27**	56.71 ± 2.40***
WS-100	206.90 ± 4.82$^{\#}$	25.57 ± 1.91	13.30 ± 1.05$^{\#}$	69.87 ± 2.02
WS-300	158.90 ± 4.35$^{\#\#}$	22.24 ± 1.28$^{\#}$	8.75 ± 0.81$^{\#\#}$	84.70 ± 2.20$^{\#\#}$
PZ-20	129.80 ± 3.18$^{\#\#}$	19.98 ± 1.15$^{\#\#}$	6.45 ± 0.64$^{\#\#}$	94.40 ± 2.60$^{\#\#}$

Table 1: *Parameters for Insulin resistance. Insulin resistance was measured after 20 weeks i.e. after 8 weeks treatment with test and standard drug.*

Values are expressed as Mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ when compared with Diabetic control group.

Effects of *Withania somnifera* on behavioural parameters of diabetic rats

Cognitive ability of diabetic animals was tested by Morris water maze (MWM) test. Escape latency time (ELT) and time spent in target quadrant (TSTQ) were taken as parameters of cognition. After assessment of ELT, it was found that animals of normal control, WS-300, PZ-20 and NC+WS-300 groups showed significantly less ELT (better learning) on day 2, 3 and 4 as compared to their ELT on day 1 (Table 2).

On day 5 (probe trail), TSTQ of diabetic control animals was significantly low ($p < 0.01$) as compared to normal control animals. Animals of WS-100 and 300 groups were found to spent significantly more time in target quadrant ($p < 0.05$) as compared to diabetic control animals (Table 2).

Groups	Escape Latency Time (ELT) (in sec)				TSTQ (in sec)
	Day 1	Day 2	Day 3	Day 4	Day 5
Normal control	72.8 ± 8.83	55.2 ± 6.24*	49.1 ± 4.32**	31.9 ± 6.33**	96.6 ± 3.66
Diabetic control	97.9 ± 11.6	91.6 ± 6.14	88.3 ± 4.7	103.3 ± 8.6	41.3 ± 3.7**
WS-100	67.5 ± 4.54	65.3 ± 5.87	55.7 ± 5.32	48.9 ± 6.31	71.2 ± 7.43$^{\#}$
WS-300	73.2 ± 10.99	56.7 ± 3.51**	34.7 ± 3.81**	42.6 ± 2.64***	64.0 ± 2.08$^{\#}$
PZ-20	81.8 ± 10.01	60.4 ± 10.44*	46.4 ± 9.88**	34.9 ± 3.70***	53.3 ± 3.18$^{\#\#}$
NC+WS-300	84.5 ± 7.57	68.4 ± 8.73*	47.8 ± 5.36**	40.0 ± 4.37***	101.3 ± 8.71

Table 2: *Escape latency time (ELT) and Time spent in target quadrant (TSTQ) in Morris Water Maze.*

Values are expressed as Mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ELT on days 2, 3 and 4 were compared with ELT on day 1, within the group. TSTQ of Diabetic control group was compared with that of Control group $^{**}p < 0.01$, while TSTQ of groups WS-100, WS-300 and PZ-20 were compared with Diabetic control group $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

Discussion

Type 2 diabetes mellitus (T2DM) is the most common metabolic disorder worldwide and one of the major cause of morbidity and mortality. Most of the diabetes cases are T2DM i.e. non-insulin dependent diabetes mellitus (NIDDM). Sedentary life style and excess

intake of fat/sugar can lead to obesity and type 2 diabetes mellitus (T2DM) accompanied by insulin resistance (IR) and β-cell dysfunction. Increasing evidences showed that 70 - 80% patients of Alzheimer's disease (AD) have abnormal levels of blood glucose or insulin. Hyperglycemia, hyperlipidemia and hyperinsulinemia are the main hallmarks of T2DM and are considered as the main reason for cognitive decline associated with diabetes [21].

Type 2 diabetes is also known as insulin resistant diabetes because insulin resistance (IR) is one of the main components of its pathophysiology. It is defined as the reduced sensitivity of the body tissues to the action of insulin and it is the major factor for the progression of T2DM. In insulin resistance (IR), there is a disruption of insulin signaling which adversely affects the glucose transport and thus inhibition of energy production due to glucose oxidation. IR was calculated by homeostasis model assessment of insulin resistance (HOMA-IR) as described by Answer., *et al*. [22] and it was found that diabetic animals have significantly more IR as compared to control animals and treatment of diabetic animals with WS and PZ for 8 weeks significantly lowered IR as compared to diabetic animals (Table 1). It is supported by previous studies by [28].

In diabetic condition, poor secretion of insulin from β cells and insufficient insulin action i.e. insulin resistance, results in hyperglycemia and hyperinsulinemia [22]. Hyperinsulinemia is the main marker for insulin resistance (IR) which is defined as the decrease in the effects of insulin to stimulate glucose uptake at normal blood concentration (Tarique A., *et al*). It is a compensatory mechanism by pancreatic β-cells in response to increase in blood glucose level. Increased concentration of glucose in the blood stimulates pancreatic β cells to secrete insulin in bloodstream.

Withania somnifera (WS) has been used in traditional system of medicine for more than 2500 years for its beneficial effects in the treatment of various disorders. It is also known as Ashwagandha and has been used widely for treating various neurological and metabolic disorders. Withaferin A is the active constituent (withanolide) and is attributed for the beneficial properties of *Withania somnifera* [24]. In this study we investigated the effects of hydroalcoholic extract of roots of *Withania somnifera* (WS) in two different doses (100 mg/kg and 300 mg/kg, orally) on metabolic and cognitive parameters of T2DM in rats. Effects of WS on T2DM induced cognitive deficit in rats was analysed by Morris water maze (MWM).

In the present study, T2DM in animals was induced by giving a combination of high fat diet and low dose streptozotocin (STZ) that mimics the natural pattern of the disease progression [16,21] which was characterized by elevated levels of fasting blood glucose, serum triglycerides, serum total cholesterol and insulin resistance (IR). IR is the main hallmark of T2DM which is indicated by significant hyperglycemia and hyperinsulinemia. Treatment of diabetic animals with hydroalcoholic extract of roots of *Withania Somnifera* (WS) for 8 weeks normalised the elevated metabolic parameters viz fasting blood glucose, triglycerides, total cholesterol and insulin as compared to untreated diabetic animals.

The results showed that HFD-STZ induced T2DM in rats which is reflected by significantly elevated levels of fasting blood glucose (FBG), serum triglycerides (TG) and serum total cholesterol (TC) as compared to normal control animals after four weeks of STZ administration (i.e. after 12 weeks of the initiation of study) as shown in figure 1-3. However, body weight of diabetic animals was found to be altered significantly.

After the confirmation of diabetes, the diabetic animals were further divided into three groups viz. WS-100, WS-300 and PZ-20 and treated according to the protocol of the study for further 8 weeks. After 8 weeks of treatment with WS (i.e. after 20 weeks of the study), FBG, TG and TC levels of the treated animals were found to be reduced significantly as compared to untreated diabetic animals as shown in figure 2-4. Body weight of the treated animals showed no significant changes as compared to diabetic animals. The results obtained were compared with the standard anti-diabetic drug Pioglitazone.

Development of type 2 diabetes deteriorated the cognitive functions of the animals as assessed by Morris water maze (MWM) test. There are several advantages of MWM over other models of learning and memory including absence of motivational stimuli such as food and water deprivation, electrical stimulations and buzzer sounds [27,28]. In morris water maze, memory is developed in animals progressively with repetitive trials that resemble the human interactions. MWM test is usually performed to test spatial learning and memory which depends on hippocampal functioning. Learning was assessed by escape latency time (ELT) which is defined as the time taken by the animal to find the hidden platform to escape swimming. During the acquisition trial the animals learn to locate a hidden platform and subsequently develop a spatial memory. To locate the hidden platform animals make an orientation map in their brain with the help of different visual cues. ELT is negatively correlated with the learning process which means shorter ELT reflects better learning in the animals. The retention of memory of is analysed by the probe trail in which the hidden platform is removed and the time spent in the target quadrant (TSTQ) is measured. TSTQ has a positive correlation with the memory i.e. more TSTQ shows good memory and vice versa [27]. The target quadrant is the quadrant where the platform was located during the acquisition trail. This model is also very helpful to analyze the reversal of amnesic effect with investigational drugs because repetitive trials confirm the progress of reversal of amnesia.

In our study, animals were subjected to the neurobehavioural evaluations by morris water maze (MWM) test. The obtained results (As shown in table 2) showed that diabetic animals had greater ELT during the period of acquisition trial (Day 1 to Day 4) i.e. they took longer time to reach the submerged platform as compared to the control animals. The results are corroborated by earlier studies which showed that administration of low dose of STZ induced peripheral and brain insulin resistant state and produced AD like behavioral changes [23]. The animals treated with WS showed significantly shorter ELT as compared to diabetic animals. Eight weeks treatment with WS also improved learning as shown by significant decrease in the escape latency time (ELT) on day 2, 3 and 4 of trial on MWM.

Also, it was observed that WS treated animals have spent more time in target quadrant on day 5 of trial (probe trial) which showed these animals have better retention of memory. Diabetic animals spent a significantly less time in target quadrant (low TSTQ) as compared to normal control animals. Treatment with WS resulted in significant increase in time spent in the target quadrant (in search of the submerged platform) in diabetic rats (Table 2). These results suggested that T2DM has a negative impact on learning as well as on memory retention that may be due to IR in the brain. WS treatment improved the acquisition and retention in the diabetic rats which may be due to reduction in the metabolic symptoms of T2DM and their impact on brain. The results were compared with the standard drug Pioglitazone.

These findings are supported by other study carried by De Felice and Ferreira, 2014 [25], which proved negative effects of diabetes on memory. Similar findings have also been reported by Dar., *et al*. 2015 who proved and concluded that WS improved the cognitive and psychomotor performances in humans [26].

Fasting blood glucose, serum triglycerides, total cholesterol, insulin and adiponectin levels of all the animals were analysed. Figure 1-3 showed that treatment of diabetic animals with WS significantly lowered the levels of FBG, TG, and TC as compared to untreated diabetic animals. Serum insulin level was found to be increased in diabetic control group shows that hyperinsulinemia was present in the diabetic animals which is the major hallmark of T2DM. Serum insulin level was decreased by the treatment with WS-300.

Adipose tissues help in regulation of insulin sensitivity by releasing different adipokines viz leptin, resistin and adiponectin in the blood. Adiponectin is the adipokine secreted in bloodstream only by adipose tissues and it acts as hormone with anti-inflammatory and insulin sensitizing properties [32]. In this study, we analysed the serum of animals for the presence of adiponectin and it was found that diabetic control animals have significantly lower levels of adiponectin as compared to control group. However, treatment with WS at the dose of 300 mg/kg and PZ significantly elevated the levels when compared with diabetic control group.

Our results are supported by previous studies which have proved that adiponectin concentration in blood is negatively correlated with development of insulin resistance [29]. According to a study by Li., *et al*. [34], adiponectin may decrease the risk of type 2 diabetes via various mechanisms like suppression of hepatic gluconeogenesis and stimulation of fatty acid oxidation, glucose uptake in muscle and insulin secretion. This indicates that WS may prevent increase in IR in progression of T2DM [30].

IR plays an important role in pathophysiology of T2DM induced neurodegenerative changes like Amnesia and Alzheimer's disease (AD) which is characterized by elevated levels of blood insulin along with reduced brain insulin levels [31,32]. Various experimental evidences suggest that brain is now recognized as insulin sensitive organ and responsible for cognitive changes due to insulin resistance. In brain, insulin receptors are mainly concentrated in hippocampus, frontal cortex (hippocampus and frontal cortex are the memory centers of the brain) in discrete concentrations where these are responsible for glucose uptake, learning and memory, food intake, weight control etc [33]. Brain insulin resistance causes the cognitive changes associated with the metabolic syndrome or T2DM [34]. Several lines of evidence suggest that brain is an insulin sensitive organ and insulin plays important role in synaptic transmission, learning and memory. Insulin may protect neurons against Aβ induced toxicity and it modulates the synaptic transmission by increasing the expression of NMDA (N-methyl-D-aspartic acid) receptors in the brain and alters long term potentiation (LTP) [35].

Earlier studies proved that the brain specific deletion of insulin receptors in mice leads to obesity and systemic insulin resistance. This indicates the importance of brain insulin signalling in the regulation of metabolic homeostasis. Insulin is transported into brain across blood brain barrier via a saturable transport system and various animal studies found that this system gets impaired in obesity and diabetes associated insulin resistance. Hence, CNS insulin levels were found to be significantly reduced in high-fat diet induced obese dogs and genetically obese rats [34]. In our study, we separated the pre-frontal cortex and hippocampus of the rats and analysed the tissue supernatant for the presence of insulin. It was observed that both prefrontal cortex and hippocampus of diabetic animals showed significantly low levels of insulin as compared to control animals whereas treatment of diabetic rats with WS at the dose of 300 mg/kg and PZ (20 mg/kg) significantly elevated the insulin levels.

The major finding in the current study is that chronic consumption of high-fat diet causes metabolic syndrome which eventually leads to memory impairments. This finding is also supported by the previous studies. For instance, administration of high fat diet for six weeks to wistar rats causes impairment in memory tested by radial arm maze [36].

The results obtained in this study showed that WS extract improved the metabolic dysfunction by reducing the levels of cholesterol, triglycerides and fasting blood sugar and also reduced systemic and brain insulin resistance. Also, it significantly improved the memory and spatial learning in cognitive impaired diabetic rats. Thus, WS can be a potential lead for the drug development for the management of T2DM and its associated cognition deficit.

Conclusion

The results of the present study indicated that root extract of *Withania somnifera* (WS) lowered the blood glucose levels, increased glucose tolerance and improved metabolic parameters (Serum triglyceride, cholesterol) in type 2 diabetic rats. Furthermore, behavioural analysis showed that WS improved the memory of the diabetic animals. Hence it can be concluded that WS could be further investigated for the clinical application for the management of type 2 diabetes mellitus.

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