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ORIGINAL ARTICLE

Grape seed proanthocyanidin extract and insulin prevents cognitive decline in type 1 diabetic rat by impacting Bcl-2 and Bax in the prefrontal cortex

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Abstract

It is frequently accepted that grape seed proanthocyanidins (GSPs) are efficient antioxidants and beneficial in improving cognitive functions. However, diabetes (T1DM)-associated declines in learning and memory and the possibilities of GSPs in overcoming this loss needs to be examined. The present study was designed to examine the correlation, if one exists, between cognitive behavior and neuronal survival in the prefrontal cortex (PFC) in streptozotocin (STZ)-induced diabetic *Wistar* rats as well as to further clarify whether the correlation exists. Also this study aimed to determine whether neurological structural changes in the PFC and pancreatic β-cells can be restored by grape seed proanthocyanidin extract (GSPE). At the end of 8 weeks, cognitive tests that rats given supplementation of GSPE and insulin had greater improvement in their spatial learning and memory skills and improved neuronal survival in the PFC and pancreatic β-cells compared to rats supplemented with either insulin or GSPE alone. Expression of Bax in the PFC was increased in the diabetic rats while Bcl-2 expression was decreased, and GSPE and insulin treatment reversed the expression of apoptotic proteins. Our findings on GSPE, a natural product, as a form of adjuvant therapy together with insulin treatment is suggestive of the existence of synergism between the two in attenuating diabetic complications in the pancreas and PFC.

Keywords Bax · Bcl-2 · Behavior · Diabetes · Grape seed proanthocyanidin · Prefrontal cortex

Introduction

Diabetes mellitus is a common metabolic disorder, characterized by hyperglycemia and associated with chronic complications such as nephropathy, angiopathy, retinopathy and peripheral neuropathy. In type 1 diabetes mellitus (T1DM), insulin production in the pancreatic islet cells is completely hindered due to a decrease in the number of islet cells. Diabetes is also associated with progressive end-organ damage in the central nervous system (Brands et al. 2005) and this complication referred to as "diabetic encephalopathy" is characterized by impaired cognitive functions involving neuronal dysfunction and neurochemical abnormalities. Neuropsychological studies have shown that patients with T1DM perform worse than patients without T1DM on several cognitive functions. Although the extent of this varies, this poor cognitive performance is inevitably noticed during childhood (Desrocher and Rove 2004), and it encompasses deficits related to intelligence, attention, processing speed, memory, and executive skills (Kodl and Seaquist 2008; Perantie et al. 2007; Northam et al. 2001 in addition to psychomotor abilities. Eventually, the progression and storage of these deficits for an extended period leads to dementia in diabetic patients (Weinger et al. 2008; Ryan et al. 2003).

During these pathological changes, damaged mitochondria develop imperfectly coupled electron transport systems and, becoming a primary source of reactive oxygen species (ROS) in the cell. Therefore, oxidative stress (OS) is widely accepted as a key mediatory process in the

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development and progression of diabetic complications, due to an increased production of free radicals (FRs) and impaired antioxidant defenses (Ceriello 2003).

Streptozotocin (STZ), a diabetes-inducer, is a powerful alkylating agent that interferes with glucose transporters and glucokinase function and induce double-strand DNA breaks (Watala et al. 2009). Grape seed proanthocyanidin extract (GSPE) is widely distributed in red grape seeds that include (+) catechin, (−)epicatechin and gallic acid. GSPE mitigates FRs and alleviates OS in the hippocampus (Asha Devi et al. 2011) and cerebral cortex (Asha Devi et al. 2006) in aging rats. Proanthocyanidins play a key role in glucose homeostasis, and the altered insulin production is also related to number of insulin producing cells (Pinent et al. 2004). GSPE is water-soluble and more easily crosses the blood-brain barrier than many other antioxidants (Ahmad 2013). Zhen et al. (2014) have reported that GSPE can improve cognitive performance in rats that are subjected to experimental seizures and exert an antihyperglycemic effect in T1DM (Pinent et al. 2004). Biochemical and structural changes in the diabetic brain are caused by a chronically increased intracellular glucose concentration (Mastrocola et al. 2005; Weigner and Jacobson 1998) and changes in the cerebral cortex –dependent neurocognitive functions (Stiles and Seaquist 2010). However, studies related to ultra structural changes in the prefrontal cortex (PFC) and β-cell destruction, which may affect the cognitive outcomes in T1DM; this may be related apoptotic factors that lead to neuronal death. Furthermore, although studies related to natural products and cognitive impairments in diabetes have been carried out, the effects of GSPE supplementation and insulin treatment on the pancreas and PFC have not yet been studied extensively.

Thus, the present study on an observed animal model of T1DM while addressing the effects of GSPE in an attempt to evaluate the responses to GSPE diabetic brain has attempted to evaluate the responses in terms of possible (i) improvement in the cognitive ability of rats with STZ-induced diabetes (ii) prevention of morphological alterations in the PFC and pancreas (iii) influence on Bcl-2 and Bax genes in the PFC (iv) effectiveness of GSPE along with adjunct insulin treatment on the aforesaid parameters.

Materials and methods

Animals

All procedures involving animals were approved by the Institutional Animal Ethics Committee (IAEC) of Bangalore University(Bangalore, India) and complied with the guidelines of the Control for the Purpose and Supervision of Experiments on Animals (CPCSEA).

Adult male Wistar rats (150–170 g) were used for the study. Three to four rats were kept in polyethylene cages and exposed to a 12 h light/dark cycle and with free access to standard food pellet (Amruth Feeds, Bengaluru), and water ad libitum.

Materials

STZ (SO130-100MG) was procured from Sigma-Aldrich, St. Louis, MO, USA. GSPE(trade name: Gravinol, GVSE 16013, 99% Purity) was obtained from Kikkoman Co. Ltd.; Noda, Japan. Gravinol is an extract from grape seeds with water and ethanol as eluents which has been concentrated followed by concentration. It contains proanthocyanidin and other components. The insulin used in this study was obtained from Novo Nordisk, India. All other chemicals were of reagent or analytical grade.

Experimental design

A total of 72 animals were divided randomly into four subgroups. Animals were categorized into six groups as follows: (1) control rats (CON); (2) control rats treated with GSPE (CON+PA); (3) STZ-induced diabetic rats (STZ); (4) diabetic rats treated with GSPE (STZ + PA); (5) diabetic rats treated with insulin (STZ + INS); and (6) diabetic rats treated with insulin and GSPE(STZ + INS + PA).

Induction of diabetes

STZ was dissolved in a 0.1 M citrate buffer (pH 4.5) and injected intraperitoneally with a single dose of 60 mg/kg of body weight following overnight fasting according to a previous data (Furman 2015; Fan et al. 2012). Diabetes was confirmed after 3 days of injection for beta-cell damage by measuring blood glucose using a glucometer (Accu-check Active) wherein the glycemic index was higher than 200–210 mg/dl.

The control group was given a buffer (vehicle), while the GSPE- supplemented animals were administered GSPE orally at a daily dose of 75 mg/kg body weight. STZ + INS and STZ + INS + PA groups were treated with insulin with a daily dose of 3 U over a period of 60 days.

Body weight and food intake were recorded weekly along with blood sugar (glucose). An oral glucose tolerance test (OGTT) was performed at 1 and 16 weeks following the induction of diabetes. Rats were fasted overnight and a glucose solution of 200 g/L was orally administered at a dose of 2 g/kg body weight. Blood glucose was measured at 0, 30, 60 and 120 min after fasting by the method described by Ding et al. (2013).

Evaluation of spatial working memory

After the confirmation of diabetes, animals ($n = 11$ per subgroup) were subjected to the T-maze in order to assess their learning performance and working memory retrieval. Win-shift strategy i.e. delayed no-match to sample (Morellini 2013) of spatial left-right discrimination tasks were employed in the T-maze (Deacon and Rawlins 2006) in this study.

The rats were first habituated in the T-maze in two phases. During the first phase (day 1, exploration), rats were allowed to explore the maze for four 5 min-trials with a given inter-trial interval (ITI) of 10 min. During each trial both arms were baited with feed as a reward. In the second phase (Days 2 and 3, habituation proper), each rat performed 10 trials with a spatial alteration delay (SAD) of 60 s in a pseudo-random sequence. During the acquisition phase (from day 4 onwards) rats were trained in accordance with the win-shift strategy. For the first trial during acquisition both arms of the maze were baited and rats received a reward by visiting either of them. Thereafter, rats were trained to avoid the previously visited arm to get the reward. Each rat had to complete the task within 120 s. After each trial, the maze was wiped with 70% ethanol to remove clues from previous trials. After 14 days of acquisition, the rats were subjected to a memory retention test. The memory retention tests were scheduled once a week for 4 weeks with 60 s SAD and 10 trials per rat; for this, rats had to complete the task within 60 s. Scoring was done manually and winning the reward was considered to indicate a correct choice. The olfactory trails between inter trials were removed by cleaning the surface of the T-Maze with 70% alcohol.

Memory

Memory was tested for rat's working and reference memory at intervals of one week over a period of 4 weeks through left- and right-discrimination learnt tasks as described earlier (Abhijit et al. 2017).

Isolation of brain regions

Following the completion of neurobehavioral studies, rats were subjected to CO_2 asphyxiation; their brains were removed quickly, washed in a 0.9% ice-cold saline and placed in cold phosphate buffer (0.1 M) prior to microdissection. The PFC was dissected from the brain between bregma 4.68 to 2.52 mm. Isolated tissue from each group were weighed and fixed overnight in a 3% buffered glutaraldehyde (pH 7.2) for 24 h for electron microscopic studies. For light microscopic studies, tissues were fixed in a 10% neutral buffered formalin and remaining tissue was stored at -80 °C until the analyses was performed. For gene expression studies, tissues were snap frozen in liquid nitrogen and stored at -80 °C for extraction of RNA.

Morphological studies

Light microscopy

Hematoxylin and eosin staining

Paraffin sections of 5 μm in thickness were deparaffinized in xylene and rehydrated through grades of ethanol by the method described by Pearse (1961). Sections were stained with hematoxylin, differentiated in 1% acid alcohol, blued in 0.2% ammonia water and rinsed in 95% alcohol. Sections were counterstained with eosin, dehydrated through graded ethanol series, cleared in xylene and mounted in DPX.

Nissl staining and neuronal quantification

Prefrontal sections of 5 μm in thickness were deparaffinized in xylene and hydrated in graded series of alcohol. Sections were further stained in 0.1% cresyl violet, dehydrated in 70% ethanol to 100% ethanol, cleared in xylene and mounted in DPX. Neuronal quantification was performed as described by Pamidi and Nayak (2014). The number of surviving neurons with their well-defined nuclei was counted while those with shrunken perikaryon and disintegrated nuclei were disqualified from quantification.

Electron microscopy

PFCs and pancreases were fixed in 3% buffered glutaraldehyde (pH 7.2) for 24 h and washed in 0.1 M phosphate buffer followed by post-fixation in 1% osmium tetroxide for 60 mino, dehydrated in graded series of distilled alcohol, cleared in propylene oxide and impregnated in 1:1 ratio of propylene oxide:Araldite resin overnight, then increased to 1:3 ratio followed by pure araldite resin for 2–3 h. Later tissues were embedded in flat embedding mould and kept in an oven at 60 °C for 48 h for polymerization (Palay and Chan-Palay 1974).

Ultramicrotomy: Tissue blocks were cut under UC6 Ultramicrotome (Leica Microsystems, Germany). Initially 1 μm thick sections were collected on a plane glass slide, stained using 1% toluidine blue and viewed under light microscope to find interested area to study light microscopic features. Later 400–500 A^0 thick ultrathin sections were collected on copper grids, stained using uranyl acetate and lead citrate as described by Frasca and Parks (1960). After proper staining, the ultrathin sections were scanned under Tecnai G2 Spirit Bio-twin Transmission Electron Microscope (FEI, Netherland) and representative areas were photographed using *Megaview III* CCD camera.

Immunohistochemistry

Pancreatic insulin immunohistochemistry (IHC) was performed using a slightly modified version of the method described by Bolkent et al. (2005) using a Histostain-Plus kit (Invitrogen, California, USA. 85–9043).

Tissue sections of the pancreases were deparaffinized, rehydrated, washed in PBS 3X, (5 min each). Endogenous peroxidase activity was inhibited by incubating the sections in a washing buffer (PBS (7):MeOH(2):0.3% H_2O_2(1). Non–specific binding sites were blocked by incubating in a blocking buffer for 30 min. The sections were incubated overnight with diluted primary antibody (1:1000 μg/ml) for insulin (Sigma-Aldrich, Missouri, United States, 084 M4769). This was followed by washing the slides 3X (5 min each) in PBS. Sections were incubated with biotinylated secondary antibody for 60 min at room temperature (RT). Sections then were washed 3X (5 min each) in PBS. Sections were incubated with an enzyme conjugate for 10 min. The substrate, diaminobenzidine tetra hydrochloride (Vector Laboratories, Burlingame, Canada. SK-4100), was added to distilled water for 5–10 min. This substrate gives a brown color at immunoreactive sites. The slides were lightly counterstained by hematoxylin to gain a good morphological identification of cells, and dehydrated by passing through ascending concentrations of alcohol and cleared by xylene. Sections were cover slipped with permanent mounting media, Vectamount (Vector Laboratories, USA,H-5000). Quantification was done by using NIH-Image J Fiji software and expressed as units.

Plasma insulin level

Fasting (8 h) and fed (2 h after having a meal) circulating insulin levels were determined by a commercial insulin ELISA kit (Rat insulin ELISA kit, Thermo Scientific, MA, USA, ERINS).

In brief, blood samples were collected from the abdominal aorta. Plasma was separated immediately from blood samples by centrifugation (3900 rpm for 20 min at 4° C). 100 μl of each standard or sample was added to 96 wells and incubated for 2.5 h at RT with gentle shaking. Then, the solutions were discarded and washed four times with wash buffer. A volume of 100 μl of biotinylated antibody was added to each well and incubated for 1 h and washed four times with a wash buffer. A volume of 100 μl of streptavidin-HRP was added to each well and incubated for 45 min at RT with gentle shaking. A volume of 100 μl of TMB substrate was added to each well and incubated for 30 min at RT in the dark with gentle shaking. Reactions were stopped by the addition of 50 μl of a stop solution to each well. The plate was evaluated in a micro- plate reader (Infinite 200 PRO, TECAN Austria GMBH) at 450 nm. A standard curve was generated by plotting the average absorbance obtained for each standard concentration along the vertical y- axis and the corresponding insulin concentration along the x- axis. The amount of insulin in each sample was determined by interpolating from the y-axis value. Sample absorbance values were multiplied by dilution factor.

Evaluation of Bcl-2 and Bax gene expression studies in prefrontal cortex

RNA isolation

Tissue samples (100 mg) were homogenized with Teflon pezzle. Total RNA was isolated using the TRIzol Reagent method (Life Technologies) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific AB-1453/A) with an oligo dT primer according to the manufacturer's instructions. The reaction volume was set to 20 μl and cDNA synthesis was performed at 42° C for 60 min, followed by reverse-transcriptase inactivation at 85° C for 5 min. Primers were obtained from Euro fins Genomics, India.

Real-time semiquantitative polymerase chain reaction

A semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out using Techno Prime system to determine the levels of Bcl-2, Bax and β-actin mRNA expressions. The RT-PCR mixture (final volume 20 μL) contained 1 μL of cDNA, 10 μL of Red Taq Master Mix 2× (Amplicon) and 1 μM of each complementary primer specific for Bcl-2, Bax and β-actin (internal control) sequences (Table 1). The samples were denatured at 94° C for 5 min, and amplified using 30 cycles of 94° C for 30 s, 55° C for 30 s, and 72° C for 1 min for Bcl-2, Bax and β-actin genes followed by a final elongation at 72° C for 10 min. The optimal numbers of cycles were selected for amplification of all three genes experimentally so that amplifications were in the exponential range and did not reach a plateau. Ten μL of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed. Quantification of the results was accomplished by measuring the optical density of the bands, using the computerized imaging program Image J. The values were normalized to β-actin intensity levels.

Statistical analyses

Data analysis was performed using GraphPad Prism 6.7 software and reported as mean ± SEM. The data was further statistically compared using a one-way analysis of variance (ANOVA) with Duncan's multiple comparison

Table 1 Sequence of primers for Bcl-2, Bax and β-actin

a	Primer pair	Sequence (5′ → 3′)	Length	Tm	GC %	Product size (bp)
Bcl-2	FP	CTGGTGGACAACATCGCTCTG	21	61.8	57.1	228
	RP	GGTCTGCTGACCTCACTTGTG	21	61.8	57.1	
Bax	FP	GACACCTGAGCTGACCTTGG	20	61.4	60	310
	RP	GAGGAAGTCCAGTGTCCAGC	20	61.4	60	
β-actin	FP	TCCTCCTGAGCGCAAGTACTCT	22	62.1	54.5	153
	RP	GCTCAGTAACAGTCCGCCTAGAA	23	62.4	52.2	

post-hoc test. Different significance statuses were assigned to groups and when compared with between groups, are represented in lower case.

Results

Body weight

A two-way ANOVA revealed diabetic condition as a significant factor for the weekly reduction in body weight ($F(5, 35) = 122.7$, $P < 0.001$). There was a significant reduction ($p < 0.05$) in body weight at the end of 4 weeks (30.8%) and 8 weeks (48.4%) in rats in the diabetic group with respect to normal control rats. However, the extent of reduction was lesser in diabetic rats that were supplemented with GSPE at the end of 4 weeks (8.46%) and 8 weeks (11.82%). In the insulin-treated diabetic rats, the reduction in body weight was by 1.23 and 3.7% at the end of 4 weeks and 8 weeks respectively of STZ administration. In the GSPE supplemented and insulin treated diabetic rats, there was insignificant reduction in body weight at both 4 weeks and 8 weeks (Table 2).

Blood glucose

Fasting blood sugar (FBS)

The FBS levels of diabetic rats were significantly higher (172.6%, $p < 0.05$) than those of controls 2 weeks following administration of STZ. At 4 and 8 weeks, blood glucose increased by 183 and 203% ($p < 0.05$) with respect to the controls. GSPE administration to diabetics increased the glucose level to a lesser extent at 8 weeks and had increased by 167% with a further reduction in the extent of increase by 146% compared to control rats (Table 3). A similar trend was seen in insulin-treated diabetic rats at 4 and 8 weeks following the induction of diabetes. However, there was insignificant change in glucose levels between GSPE-supplemented diabetic and unsupplemented control rats.

Post prandial blood sugar (PPBS)

PPBS in the diabetics showed significant increases of 183 and 200% ($p < 0.05$) compared to control rats at 4 and 8 weeks of diabetes induction. The extent of the increases in glucose was reduced by 183 and 160.5% at 4 and 8 weeks in animals supplemented with GSPE. Insulin-treated diabetic rats, however, exhibited lesser extent of glucose elevation and were by 160 and 153% higher than controls at 4 and 8 weeks after diabetes induction. GSPE supplementation combined with insulin treatment to diabetic animals resulted in the lowest elevation in glucose at 4 weeks (155.7%) and 8 weeks (148%) (Table 3).

Overall, the extent of increases in blood glucose, FBS, and PPBS, in the diabetics is summarized as: (STZ + PA) > (STZ + INS) > (STZ + PA + INS) which is suggestive of a synergistic effect of GSPE and insulin in suppressing the elevation of glucose levels in diabetic animals through the 8- week treatment of insulin and oral supplementation of GSPE.

Oral glucose tolerance test

Table S1 shows the glucose tolerance of the control and experimental groups at week 1($F(5,15) = 4.254$, $p < 0.001$) and at week 8($F(5,15) = 5.547$, $p < 0.001$). Diabetic rats exhibited significantly ($p < 0.05$) higher values compared to control rats in the tests at 1 week and 8 weeks. GSPE- supplemented diabetic rats presented significantly ($p < 0.05$) lower glucose levels at the two test periods with respect to the unsupplemented diabetic rats. A similar trend of reduced glucose levels in the insulin-treated rats was seen with respect to the untreated diabetic rats. A combination of GSPE and insulin resulted in the lowest glucose levels compared to either GSPE or insulin alone.

Food intake

Mean food intake between the controls and experimental groups throughout the study showed significant differences ($F(5, 35) = 6.083$, $P < 0.001$).

Diabetic rats showed higher food intake compared to the control rats. However, GSPE-supplemented and insulin-treated diabetic rats presented lower food intake with respect to untreated

Table 2 Body weight in response to GSPE and insulin treatment in streptozotocin-induced diabetic rats

Body weights (gm)						
Week	CON	CON+PA	STZ	STZ + PA	STZ + INS	STZ + INS + PA
1	161.6 ± 2.3^{ad}	166.4 ± 1.6^{ad}	148.3 ± 2.6^{b}	149.1 ± 3.0^{b}	159.6 ± 3.3^{d}	160.1 ± 1.5^{ad}
2	$164.\ 1 \pm 4.2^{ad}$	172.5 ± 3.0^{a}	140.4 ± 2.3^{b}	152.7 ± 2.5^{c}	161.0 ± 3.4^{d}	162.1 ± 2.6^{d}
3	171.9 ± 2.1^{ad}	180.0 ± 2.3^{a}	135.6 ± 1.4^{b}	159.4 ± 3.5^{c}	169.3 ± 2.3^{d}	171.5 ± 3.2^{d}
4	184.3 ± 1.6^{ad}	187.7 ± 3.3^{a}	127.4 ± 1.3^{b}	168.7 ± 1.6^{c}	171.8 ± 2.6^{d}	174.3 ± 2.7^{d}
5	188.1 ± 1.4^{a}	192.1 ± 1.5^{a}	120.5 ± 1.1^{b}	165.4 ± 1.1^{c}	176.5 ± 1.5^{da}	182.4 ± 1.9^{ad}
6	192.4 ± 1.5^{a}	198.7 ± 2.0^{a}	114.8 ± 1.1^{b}	170.8 ± 2.3^{c}	180.8 ± 1.7^{d}	187.3 ± 1.6^{ad}
7	203.7 ± 1.2^{a}	210.6 ± 1.2^{a}	110.0 ± 1.0^{b}	174.8 ± 2.8^{c}	189.4 ± 1.5^{d}	196.8 ± 2.8^{ad}
8	210.6 ± 1.3^{ad}	220.8 ± 2.1^{a}	108.7 ± 1.4^{b}	185.7 ± 2.5^{c}	202.8 ± 1.6^{d}	208.4 ± 1.2^{d}

CON, control; CON+PA, GSPE supplemented control; STZ, STZ-induced diabetics; STZ+PA, GSPE supplemented diabetics; STZ + INS, diabetics treated with insulin; STZ + INS + PA, diabetics treated with INS and GSPE. GSPE, grape seed proanthocyanidin extract. Values are mean ± SEM (n = 7). Significance was calculated by two-way ANOVA using GraphPad Prism 6 followed by Bonferroni comparison test. Groups sharing dissimilar lower case are significant at $p < 0.05$

diabetic rats. Food intake was significantly reduced in insulin-treated and GSPE-supplemented rats with respect to diabetic rats and compared to single treatments as shown in Table S2.

Behavioral studies

Assessment of changes in learning

Further, in order to examine the possible effect of GSPE and insulin treatment in the animal's cognitive ability, learning and memory tests were conducted in a T-maze.

The two-way ANOVA revealed significant differences between the control and experimental groups on day 4 ($F(5,36) = 35.73$, $P < 0.001$) and day 8 ($F(5,36) = 63.14$, $p < 0.001$). Percent acquisition in the T-maze is represented in Fig. 1. As shown in Fig. 1a, on day 4, control rats showed 45% correct choices while supplemented controls displayed 54% correct choices. Diabetic rats made only 27% correct choices; this increased to 38% when supplemented with GSPE ($p < 0.0001$). Diabetic rats with insulin -treatment displayed the same extent of correct choices ($P < 0.001$) as that of their untreated counterparts. Moreover, even more correct choices

Table 3 Blood sugar in response to GSPE and insulin treatment in streptozotocin-induced diabetic rats

Week	CON	CON+PA	STZ	STZ + PA	STZ + INS	STZ + INS + PA
	FBS					
	FBS (mg/dl)					
2	92.5 ± 7.6^{a}	92.0 ± 7.2^{a}	252.2 ± 16.0^{bc}	249.0 ± 10.1^{bc}	248.2 ± 6.7^{bc}	245.7 ± 6.1^{b}
4	92.5 ± 7.0^{a}	94.0 ± 6.5^{a}	262.2 ± 15.2^{bc}	247.7 ± 16.9^{b}	243.7 ± 11.9^{b}	236.0 ± 4.7^{b}
6	94.0 ± 8.0^{a}	93.0 ± 8.9^{a}	278.0 ± 6.6^{b}	241.0 ± 8.5^{c}	240.5 ± 11.6^{c}	230.7 ± 8.5^{c}
8	94.5 ± 9.1^{a}	94.0 ± 6.1^{a}	287.0 ± 8.8^{b}	233.0 ± 9.1^{c}	230.0 ± 6.3^{c}	227.0 ± 5.9^{c}
10	91.7 ± 7.8^{a}	94.5 ± 9.7^{a}	295.2 ± 6.9^{b}	228.7 ± 6.1^{c}	226.2 ± 12.2^{c}	220.0 ± 11.5^{c}
	PPBS (mg/dl)					
2	126.75 ± 9.29^{a}	123.7 ± 7.6^{a}	343.5 ± 9.9^{bc}	338.7 ± 12.9^{b}	335.2 ± 10.3^{bc}	332.0 ± 9.3^{bc}
4	125.5 ± 8.2^{a}	124.0 ± 6.5^{a}	355.7 ± 11.1^{b}	335.7 ± 7.2^{bc}	326.0 ± 10.5^{bc}	321.0 ± 6.2^{c}
6	122.5 ± 8.4^{a}	121.7 ± 7.7^{a}	365.7 ± 7.4^{b}	330.0 ± 5.8^{dc}	320.0 ± 8.7^{dc}	317.2 ± 12.7^{c}
8	125.5 ± 5.3^{a}	124.0 ± 13.0^{a}	376.2 ± 9.3^{e}	327.0 ± 5.2^{bc}	318.2 ± 6.7^{bc}	312.0 ± 8.1^{c}
10	122.2 ± 8.4^{a}	123.2 ± 5.3^{a}	418.0 ± 9.1^{e}	321.2 ± 8.77^{bc}	308.7 ± 6.4^{bc}	300.5 ± 8.8^{c}

CON, control; CON+PA, GSPE supplemented control; STZ, STZ-induced diabetics; STZ+PA GSPE supplemented diabetics; STZ + INS, diabetics treated with insulin; STZ + INS + PA, diabetics treated with INS and GSPE. GSPE, grape seed proanthocyanidin extract. Values are mean ± SEM (n = 7). Significance was calculated by two-way ANOVA using GraphPad Prism 6 followed by Bonferroni comparison test. Groups sharing dissimilar lower case are significant at $p < 0.05$

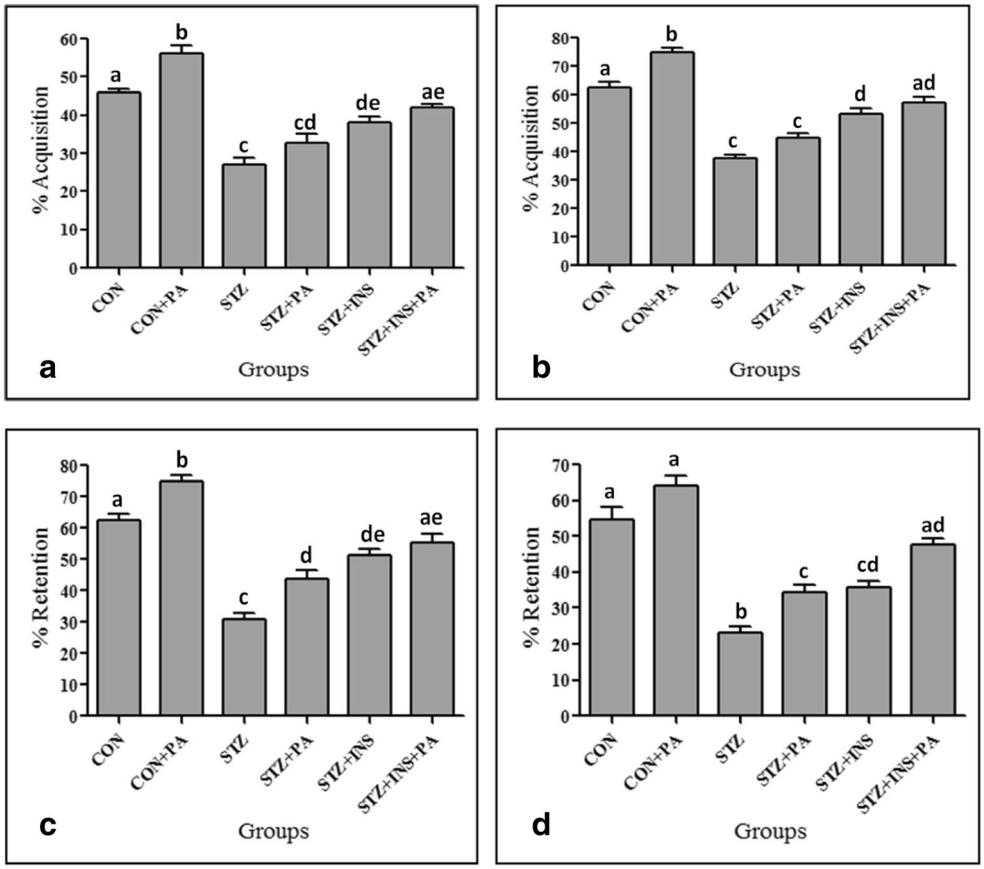

Fig. 1 Effects of GSPE and insulin on cognitive behaviors in a T-maze in diabetic rats. Acquisition is represented in terms of % correct choice at 4 days (**a**) and 8 days (**b**) of learning and at 2- (**c**) and 4-(**d**) weeks of spatial working memory tests. CON, control; CON(PA), GSPE-supplemented control; STZ, STZ-induced diabetics; STZ+(PA), GSPE-supplemented diabetics; STZ + INS, diabetics-treated with insulin; STZ + INS + GSPE, diabetics treated with insulin and GSPE. GSPE, grape seed proanthocyanidin extract. Values are mean ± SEM of 7 rats/group. Values are mean ± SEM ($n = 7$ rats/group). Significance was tested by Tukey's Multiple Comparison Test and is represented in lower case. Groups sharing different letters are significant ($p < 0.05$)

(40%)($P < 0.001$) were made with a combined-insulin treatment and GSPE supplementation.

On day 8 (Fig. 1b), acquisition was improved in the control rats, showing 60% correct choices while diabetics showed only a slight improvement (38%). However, GSPE-treated and insulin-treated diabetic rats made 48 and 58% correct choices ($P < 0.05$) respectively while insulin treatment combined with GSPE supplementation resulted in rats making 58% correct choice ($P < 0.05$).

Memory

GSPE and insulin treatment either singly or in combination in diabetic rats showed significantly improved memory in terms of correct choice during the memory test in the second week ($F(5, 36) = 52.33$ $P < 0.001$) and fourth week ($F(5, 36) = 36.61$, $P < 0.001$) (Fig. 1c, d). STZ-induced diabetic rats showed significantly reduced memory retrieval compared to the non-diabetics when tested 2 weeks after the last learning session. GSPE-supplemented rats improved their memory retention to a similar extent as insulin- treated ones, as shown in Fig. 1c.

At week 4, memory retention was lowered in all groups compared to those that were tested inweek 2 (Fig. 1d). Compared to the untreated diabetic animals, GSPE-supplemented and insulin-treated animals showed an almost equal extent of improvement in memory retention. A combination of INS and GSPE resulted in an improvement in memory retention, but this was lower compared to that seen during the second week.

Morphology of pancreas and prefrontal cortex

Following the behavior tests, anatomical changes in the PFC, region involved in cognitive skills were studied along with insulin-secreting β-cells.

Pancreas

Light microscopy

Figure 2 displays photomicrographs of pancreatic tissue stained with H & E. There was a reduction in the number of islets in the diabetic animals compared to the controls, and diabetics supplemented with GSPE showed improvements in this variable. Pancreatic tissues from normal non-diabetic rats and those supplemented with GSPE showed regular islets with clustered beta cells located centrally (Fig. 2a). In the diabetic rats, islets

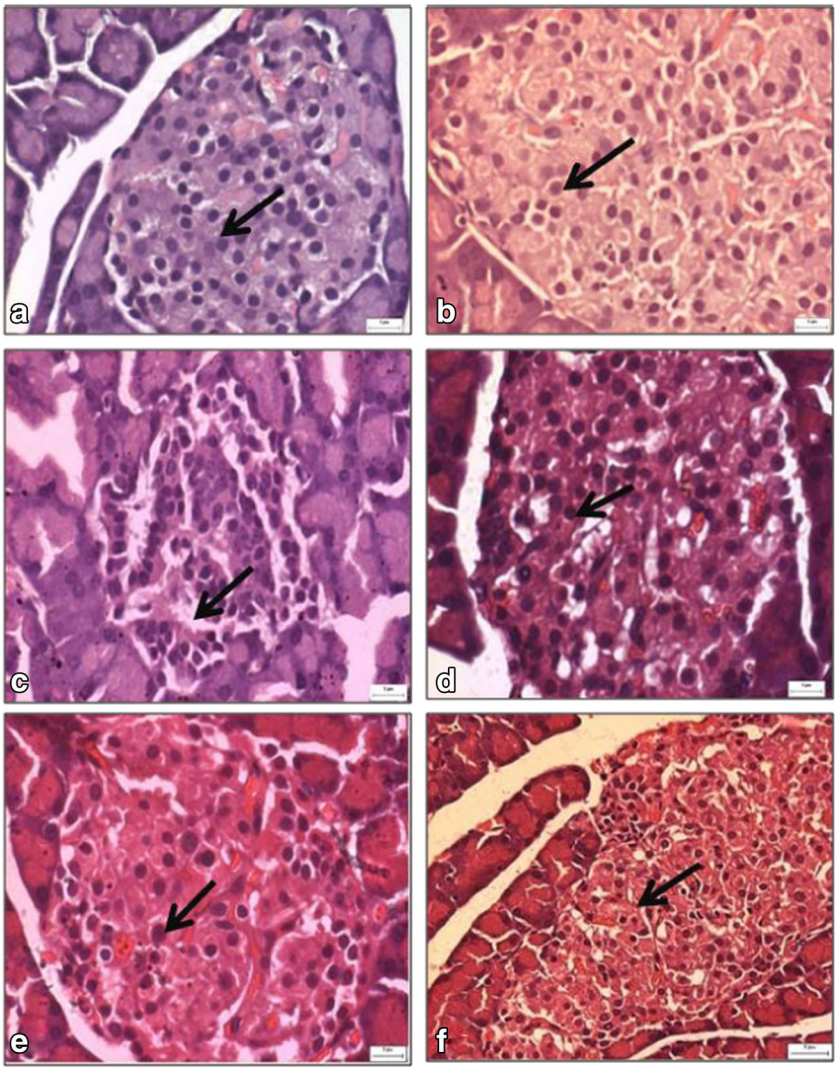

Fig. 2 Photomicrographs of rat pancreatic tissue stained with hematoxylin and eosin. **a** CON, control; **b** CON+PA, GSPE supplemented control; **c** STZ, STZ-induced diabetics; **d** STZ+PA, GSPE supplemented diabetics; **e** STZ+INS, diabetics treated with insulin; **f** STZ+INS+PA, diabetics treated with INS and GSPE. X 40, Scale bar = 5 μm

showed disintegrated β-cells (Fig. 2c) while GSPE restored the normal morphology of β-cells (Fig. 2d). Pancreatic sections from insulin-treated diabetic animals had near- normal appearance of islets (Fig. 2e). However, a combination of GSPE and insulin was more effective in restoring the normal structure of pancreases in the diabetic animals (Fig. 2f).

Ultrastructure of pancreas

The pancreases from control and GSPE-supplemented rats showed normal morphology (Fig. 3a, b) with normal mitochondria and ER and a large number of secretory granules in the β-cells. In the diabetic rats, disrupted secretory granules and ER dilation were seen (Fig. 3c). Secretory granules has increased and normal mitochondria in the β-cell were noticed in the GSPE-supplemented diabetic animals (Fig. 3d). In the insulin-treated (Fig. 3e), and GSPE- supplemented and treated with insulin treated (Fig. 3f) exhibited abundant secretory granules, normal mitochondria and RER.

Prefrontal cortex

Light microscopy

Significant loss in the neuronal cell body was seen in diabetic PFCs (Fig. 4c). However, GSPE supplemented rats showed neurons with clear nuclei (Fig. 4d). Insulin improved the number of surviving neurons (Fig. 4e). However, diabetic rats with insulin treatment and GSPE supplementation (Fig. 4f) showed an even greater number of neurons with clear nuclei.

Ultrastructure

Mitochondria in the control rats (Fig. 5a) and those treated with GSPE (Fig. 5b) were intact and compact with well defined cristae and membranes. In contrast to these,

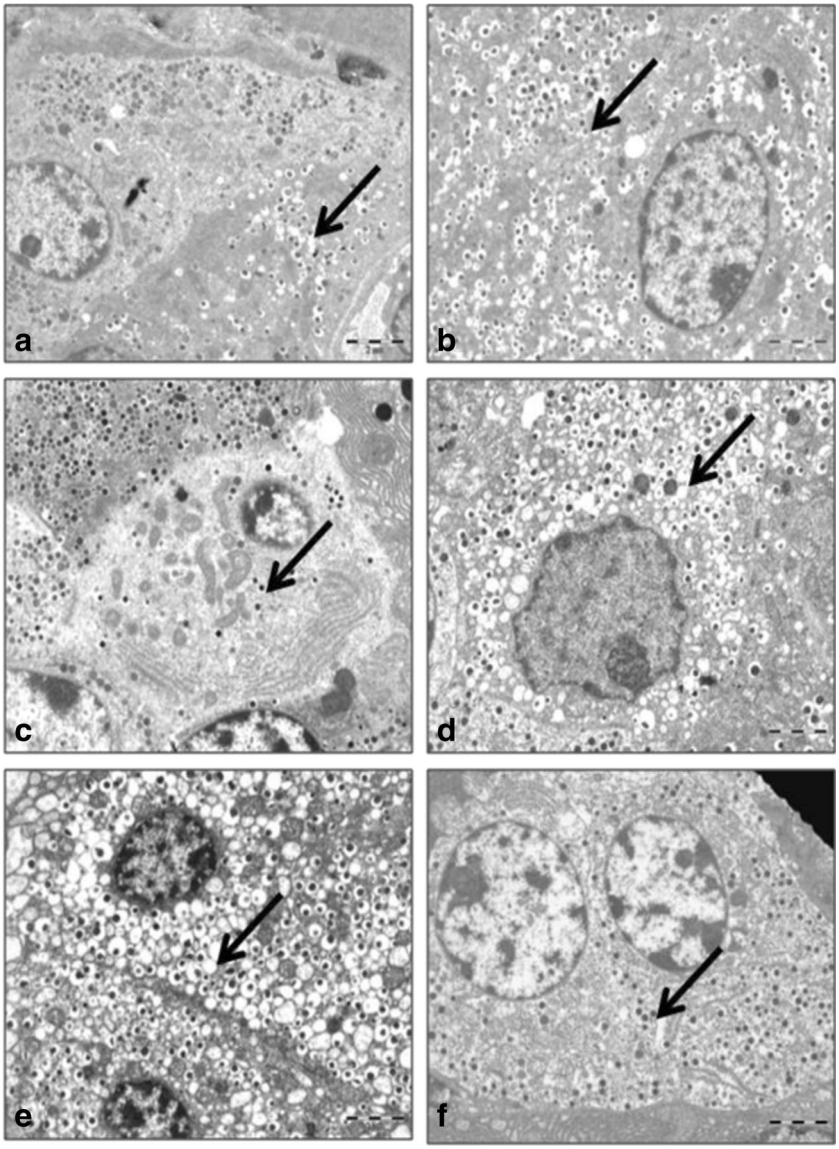

Fig. 3 Electron micrographs of pancreatic β-cells stained with uranyl acetate-lead citrate in. **a** CON, control; **b** CON+PA, GSPE supplemented control; **c** STZ, STZ-induced diabetics; **d** STZ+PA, GSPE supplemented diabetics; **e** STZ+INS, diabetics treated with insulin; **f** STZ+INS+PA, diabetics treated with INS and GSPE. X 23000 (Original magnification)

mitochondria were irregular, enlarged, and vacuolated with damaged cristae in the diabetic rats (Fig. 5c). GSPE supplementation resulted in more intact mitochondria with regular cristae compared to the diabetic ones (Fig. 5d). In the insulin-treated diabetic rats, a lesser extent of mitochondrial damage was evident (Fig. 5e). A combination of insulin treatment and GSPE supplementation resulted in more normal mitochondria and RER (Fig. 5f).

Neuronal quantification

Two-way ANOVA revealed significant differences in neuronal survival in the PFC between the control and experimental groups at week 1[$F(5,30) = 31.31$, $P < 0.001$]. Figure 6 shows neuronal quantification wherein the number of surviving neurons was significantly reduced in the diabetic group compared to the control group, while GSPE improved the number of surviving neurons. A daily dose of insulin and GSPE resulted in better survivability of the neurons compared to either of them alone.

Pancreatic insulin immunoreactivity

Figure 7 represents photomicrographs of insulin immunoreactivity in the pancreatic islets. In the control group (Figs. 7a and 8a) and GSPE-treated control animals, islets displayed normal β cells (Figs. 7b and 8a) while in the diabetic (Figs. 7c and 8a), the number of cells displaying insulin immunoreactivity was lower than in rats supplemented with GSPE (Figs. 7d and 8a). However, daily insulin treatments and GSPE supplementation resulted in levels almost similar to the controls (Figs. 7f and 8a).

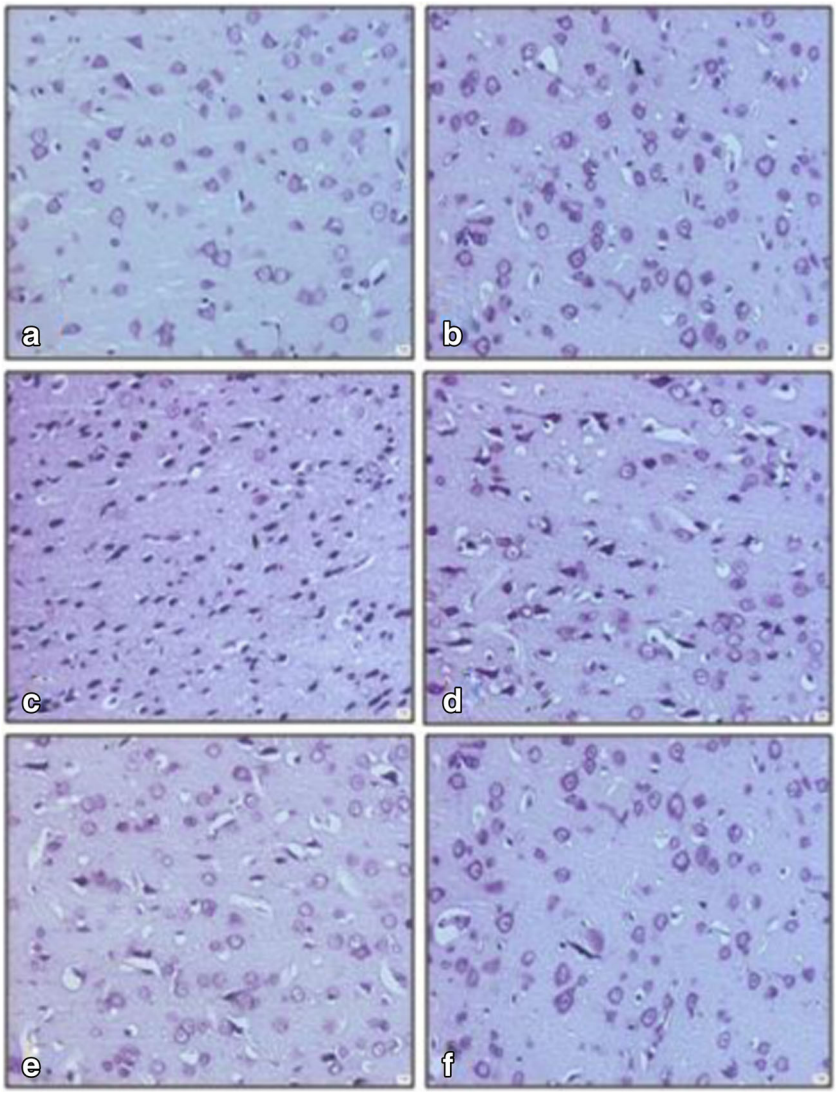

Fig. 4 Microphotographs of rat prefrontal cortex stained with cresyl violet for Nissl substance. **a** CON, control; **b** CON+PA, GSPE supplemented control; **c** STZ, STZ-induced diabetics; **d** STZ+PA, GSPE supplemented diabetics; **e** STZ+INS, diabetics treated with insulin; **f** STZ+INS+PA, diabetics treated with INS and GSPE. X 40

The one-way ANOVA showed significant effects of treatments ($F(5,12) = 41.61$, $P < 0.0001$) on the insulin immunoreactivity of the pancreas. Immunoreactivity of the islets was low in the diabetics compared to the controls while GSPE supplementation resulted in improved reactivity. In the pancreatic islets of GSPE- supplemented and insulin- treated diabetics, the immunoreactive of β cells was increased compared with either insulin or GSPE alone (Fig. 8a).

Plasma insulin

The one-way ANOVA revealed significant differences between the treatments on the fasting ($F(5,12) = 33.62$, $P < 0.001$) and post-prandial ($F(5,12) = 33.62$, $P < 0.001$) insulin levels. In the diabetic rats, plasma insulin was reduced significantly (Fig. 8b, c) while these levels improved in the GSPE-supplemented diabetics. Insulin treatment alone or insulin treatment in combination with GSPE supplementation was more effective in rising insulin levels with respect to the diabetic animals. Interestingly, the plasma insulin levels when checked at 8 weeks following STZ administration could be related to the high glucose tolerance of the diabetics.

Evaluation of Bcl-2 and Bax expression in prefrontal cortex

In order to study whether the observed deficits in cognitive skills and decreased neuronal survival in the PFC is related to pro-and anti-apoptotic gene expressions, a set of diabetic and treated rats were tested for Bcl-2 and Bax expressions.

The level of Bcl-2 expressed in the PFC of diabetic rats was significantly lower compared with control group (Fig. 9a, lane 4). However, GSPE treatment increased Bcl-2 expression (Fig. 9a, lane 4). Compared to the control group, Bax expressions were increased in the diabetic

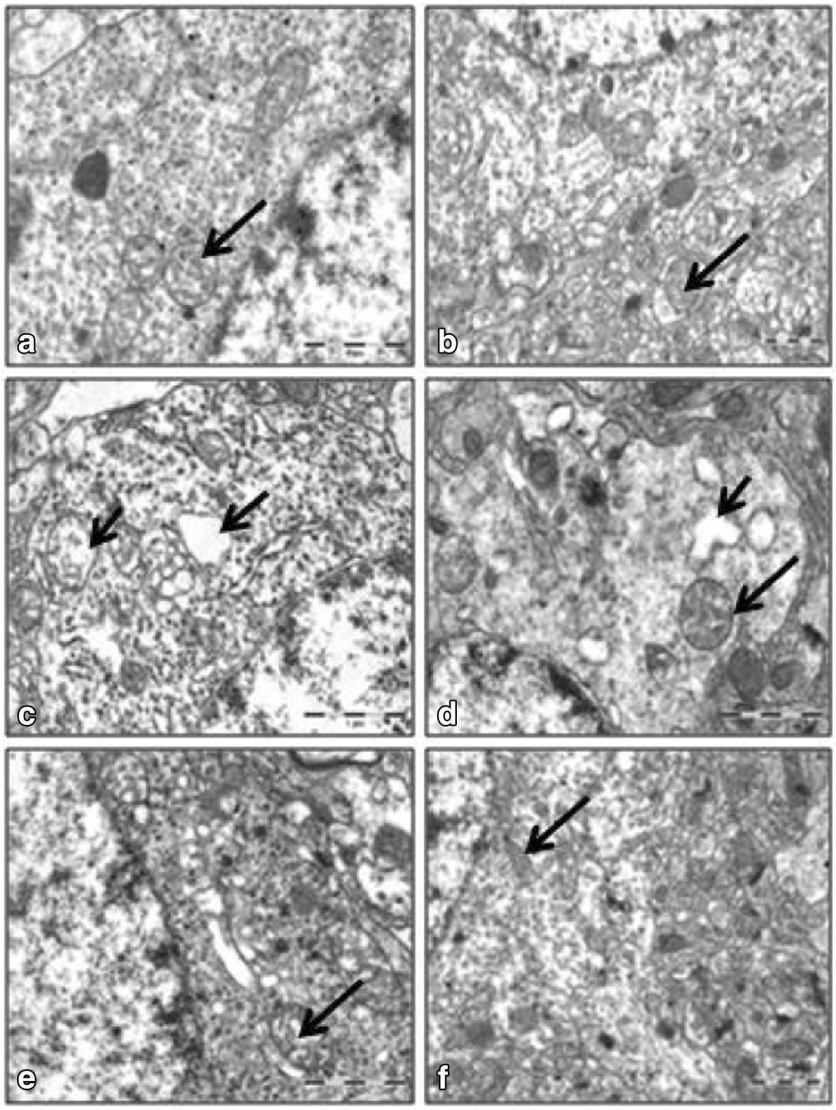

Fig. 5 Electron microphotographs of rat prefrontal cortex stained with uranyl acetate-lead citrate. **a** CON, control; **b** CON+PA, GSPE supplemented control; **c** STZ, STZ-induced diabetics; **d** STZ+PA, GSPE supplemented diabetics; **e** STZ+INS, diabetics treated with insulin; **f** STZ+INS+PA, diabetics treated with INS and GSPE. Arrows indicate normal mitochondria and short arrow indicate swollen mitochondria in neuronal body. X 23000 (Original magnification)

group (Fig. 9b, lane 3). Interestingly, GSPE was shown to increase Bcl-2 (Fig. 9a, lane 4) and simultaneously decrease Bax (Fig. 9b, lane 4) expressions in the diabetic group. The level of expression of Bcl-2 in insulin-treated (Fig. 9a, lane 5) diabetic rats was higher than in the untreated rats (Fig. 9a, lane 3). The level of Bax expression was lower in the insulin-treated diabetic group (Fig. 9b, lane 5). Interestingly, GSPE in combination with insulin-treatment revealed a greater expression of Bcl-2(Fig. 9b, lane 6) with negligible levels of Bax (Fig. 9b, lane 6).

Figure 10 shows the Bax/Bcl-2 ratio in the PFC. The ratio was significantly elevated in the diabetic group compared to the control group and all other experimental groups. Further, GSPE and insulin-treated groups showed significant decreases in the ratio compared to the diabetic group, although the changes were insignificant between GSPE supplemented and insulin- treated groups. The lowest ratio of pro-apoptotic and anti-apoptotic protein expression were seen with a combined insulin and GSPE treatment.

Correlation studies

The Pearson correlation test was performed to determine if any correlation, exists between the Bax and Bcl-2 expression and the expression of Bax and Bcl-2 individually with memory retention in the PFC of experimental groups following GSPE and insulin treatments. Fig.S1 is a graphical representation of the above description. Fig.S1 (A) depicts negative correlation between Bax and Bcl-2 ($r = -0.7020$). The existence of a positive correlation was seen between % memory retention and fold expression of Bcl-2 at week 2 ($r = +0.511$) and week 4 ($r = +\ 0.500$); these are depicted in Figs.S1 (B) and (D) respectively. The occurrence of negative correlations between fold expression of Bax and % memory retention at

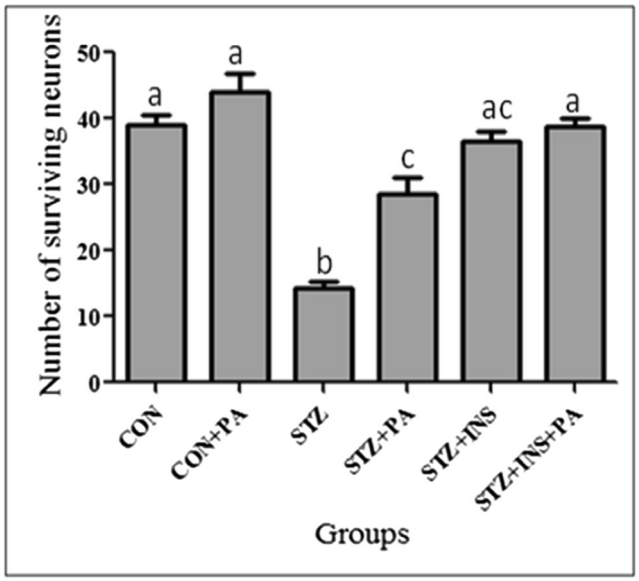

Fig. 6 Neuronal survival in the rat prefrontal cortex as a function of GSPE supplementation and insulin treatment in diabetic rats. Values are mean ± SEM (n = 3 rats/group). Significance was tested by Tukey's Multiple Comparison Test and is represented in lower case. Groups sharing different letters are significant ($p < 0.05$)

week 2 ($r = -0.650$) and week 4 ($r = -0.55$) are shown in Figs.S1 (C) and (E) respectively.

The correlation between neuronal survival and pro-and anti-apoptotic factors is represented in Fig.S2. A positive relation was seen between the number of surviving neurons and fold expression of Bcl-2 ($r = +0.703$) while neuronal survival correlated negatively with BAX ($r = -0.762$).

Discussion

In our study, GSPE and insulin, both singly, and combined were tested in vivo to evaluate their role in the progression in the cognitive decline of diabetic rats. The observed results on body weight loss accompanied by elevated glucose levels and glucose tolerance in the diabetic rats, despite increased food intake, may be a consequence of depleted glucose levels in the organs resulting from reduced insulin synthesis (Moussa 2008) and an elevated catabolism of carbohydrates (Novikova et al. 2013; King 2012; Reyes et al. 2006). GSPE alone when administered orally was effective in reducing FBS, PPBS and glucose tolerance levels and also resulted in an improvement in body weight as much as an insulin treatment. Our result on high glucose tolerance in the diabetic rats is concomitant with lowered plasma insulin and pancreatic immunoreactive insulin. Interestingly, a combination of GSPE and insulin significantly altered the above parameters, while insulin and GSPE alone altered to a lesser extent. Zhen et al. (2014) have shown proanthocyanidins as natural antioxidants with insulin mimetic action. On the contrary, Al-Awwadi et al. (2004) have reported no impact of polyphenolic extracts on

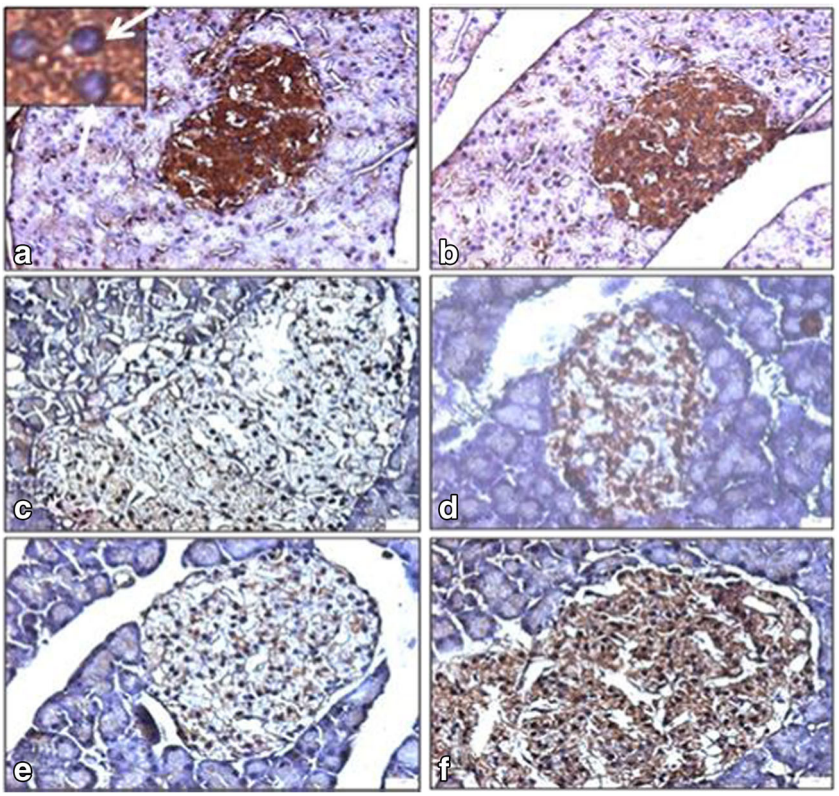

Fig. 7 Immunohistochemical reactivity of insulin secreting ß-cells in the pancreas. **a** CON, control; **b** CON+PA, GSPE supplemented control; **c** STZ, STZ-induced diabetics; **d** STZ+PA, GSPE supplemented diabetics; **e** STZ+INS, diabetics treated with insulin; **f** STZ+INS+PA, diabetics treated with INS and GSPE.

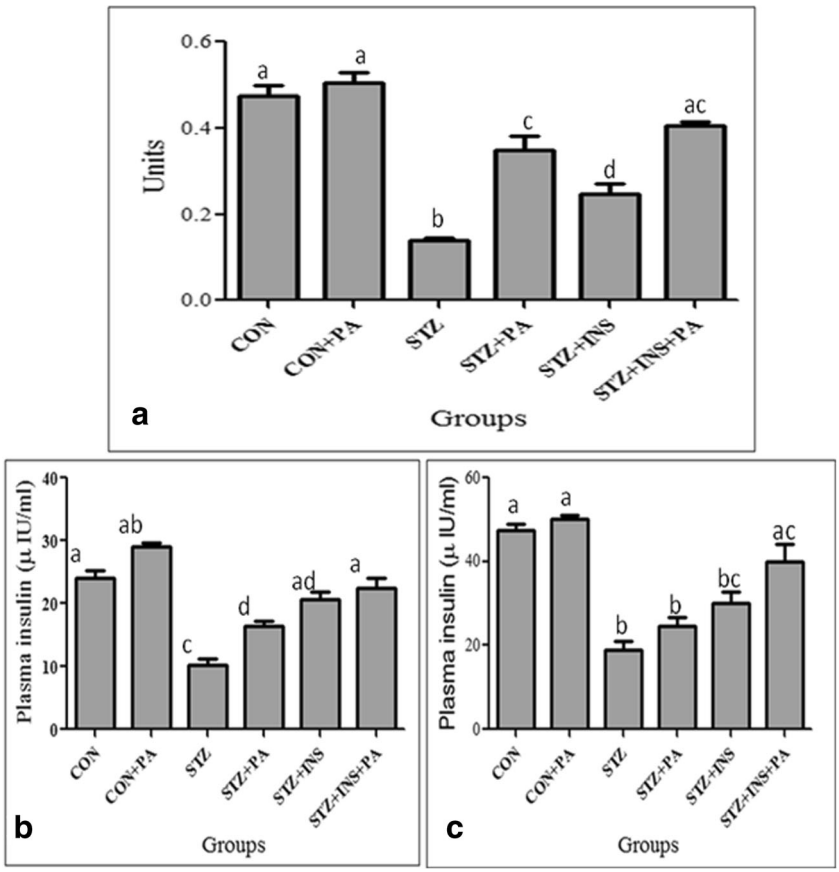

Fig. 8 Quantification of immunoreactivity of insulin in the pancreas. The optical density was analyzed, and the results are shown in the bar graphs (**a**). Plasma fasting insulin levels (**b**) and postprandial insulin levels(**c**). Values are mean ± SEM ($n = 3$ rats/group). Significance was tested by Tukey's Multiple Comparison Test and is represented in lower case. Groups sharing different letters are significant ($p < 0.05$)

plasma insulin levels in STZ-induced diabetic rats. Our results on restoration of normal glucose levels due to GSPE may be related to lesser destruction of β-cells in the diabetic rats and is in accordance with findings reported by Kao (2009) and El-Alfy et al. 2005. In STZ-induced T1DM, DNA alkylation is the primary source of β-cell destruction apart from the increased effects of reactive oxygen species on the pancreas (Lenzen 2008). The present result on the improved number of β-cells in diabetic animals treated with combined insulin and GSPE can be related to earlier reports on cell regeneration following immediate treatment with insulin for hyperglycemia (Thulesen et al. 1997) and of GSPs on restoration of normal architecture of beta cells (El-Alfy et al. (2005).

The present study, ultrastructurally demonstrates a reduced number of insulin secretory granules in the β- cell along with disrupted and vacuolated mitochondria in diabetic rats. GSPE supplementation as an adjunct therapy with a daily low dose of insulin treatment was effective in reversing these changes. Further, our observations on increased Nissl-positive neurons with condensed cytoplasm and pyknotic nuclei in the PFC of diabetic rats is suggestive of apoptotic cells (Hernández-Fonseca et al. 2009) causing a decline in the cognitive ability in STZ-induced diabetes (Artola 2008). The present findings on the gluoconeurotoxicity induced by STZ in the neurons of PFC are comparable to the earlier findings of Tomlinson and Gardiner (2008) on hyperglycemic-induced neurotoxicity in

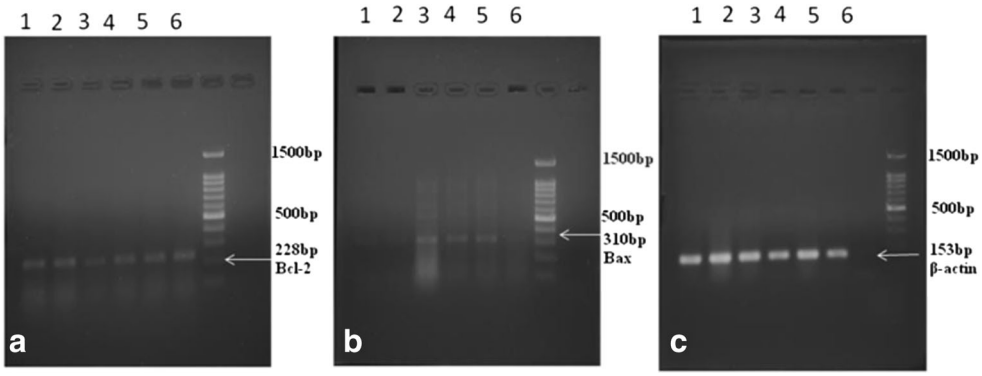

Fig. 9 **a** Amplification of antiapoptotic (Bcl-2), **b** apoptotic (Bax) and **c** housekeeping gene (β-actin) expression in the prefrontal cortex. (1), CON; (2), CON+PA, control supplemented with GSPE; (3), STZ, STZ-induced diabetics; (4), STZ+PA; GSPE supplemented diabetics; (5), STZ+INS, diabetics treated with insulin; (6), STZ+INS+PA, diabetics treated with INS and GSPE.

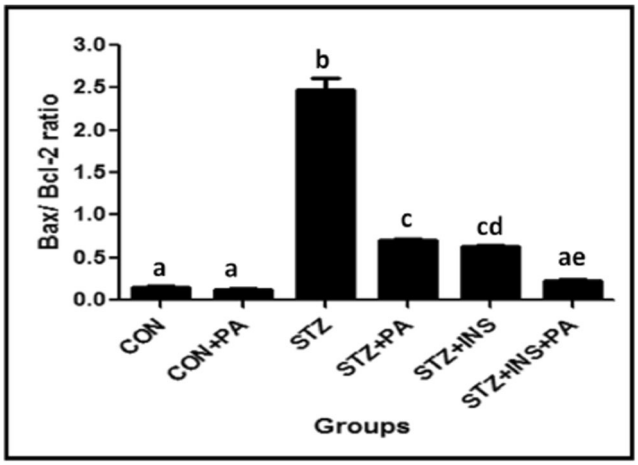

Fig. 10 Bax/Bcl-2 ratio in the prefrontal cortex of CON, control; CON+PA, GSPE supplemented control; STZ, STZ-induced diabetics; STZ+PA, GSPE supplemented diabetics; STZ+INS, diabetics treated with insulin; STZ+INS+PA, diabetics treated with INS and GSPE. The data is expressed as mean ± SEM of 3 rats per group. Significance was calculated by one-way ANOVA and post-hoc test was done in accordance with Tukey's multiple comparison using GraphPad Prism 6 and significance is represented at $p < 0.05$

rats subjected to STZ. Significant recovery in the insulin and GSPE treated diabetics is comparable to the report on alleviated stress with environmental enrichment exposures in STZ-treated diabetic rats (Pamidi and Nayak 2014) and of GSPE in protecting pyramidal neurons of the cerebral cortex in STZ-induced diabetes through modulation of advanced glycation end products and NF-kβ p65(Lu et al. 2010).

Animal models of T1DM show different extents of spatial learning and memory loss (Popovic et al. 2001) with the involvement of the cortex and hippocampus (Parihar et al. 2004). In this study, the learning and memory abilities of diabetic rats were evaluated by a T-maze test that is suitable for exploratory and spatial cognition assessments (Masso et al. 2017). Our data indicates that T1DM rats develop working memory and learning deficiencies that areconsistent with previous reports on behavior test in a Y-maze (Mirshekar et al. 2011). Interestingly, our results show that insulin treatment and GSPE supplementation improves acquisition and retention in STZ-treated diabetic animals compared to non-diabetic controls. These findings suggest a decrease in Bax with concomitant increases in Bcl-2 levels in the PFC.

In summary, our experimental evidence on animal studies indicates a role of GSPE, a natural product, as an adjunct form of therapy with a low insulin dose to alleviate the over expression of apoptotic proteins and the prevention of cognitive declines in T1DM rats. Studies on adult diabetic animals, suggest that GSPE is a possible adjunct therapy to be used along with insulin to alleviate mild cognitive declines in adult human subjects that progress with cognitive impairment in the later years of life.

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Compliance with ethical standards

Conflict of interest All authors state there is no conflict of interest.

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