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Suppression of NF-κβ signaling pathway by tocotrienol can prevent diabetes associated cognitive deficits

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ABSTRACT

Objective: The etiology of diabetes associated cognitive decline is multifactorial and involves insulin receptor down regulation, neuronal apoptosis and glutamatergic neurotransmission. The study was designed to evaluate the impact of tocotrienol on cognitive function and neuroinflammatory cascade in streptozotocin-induced diabetes.

Research design and method: Streptozotocin-induced diabetic rats were treated with tocotrienol for 10 weeks. Morris water maze was used for behavioral assessment of memory. Cytoplasmic and nuclear fractions of cerebral cortex and hippocampus were prepared for the quantification of acetylcholinesterase activity, oxidative–nitrosative stress, tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), NFκβ and caspase-3.

Results: After 10 weeks of streptozotocin injection, the rats produced significant increase in transfer latency which was coupled with enhanced acetylcholinesterase activity, increased oxidative–nitrosative stress, TNF-α, IL-1β, caspase-3 activity and active p65 subunit of NFκβ in different regions of diabetic rat brain. Interestingly, co-administration of tocotrienol significantly and dose-dependently prevented behavioral, biochemical and molecular changes associated with diabetes. Moreover, diabetic rats treated with insulin–tocotrienol combination produced more pronounced effect on molecular parameters as compared to their per se groups.

Conclusions: Collectively, the data reveal that activation of NFκβ signaling pathway is associated with diabetes induced cognitive impairment and point towards the therapeutic potential of tocotrienol in diabetic encephalopathy.

1. Introduction

Emerging evidence reveals that diabetes adversely affects the central nervous system. Both acute and chronic metabolic and vascular disturbances can impair the functional and structural integrity of the brain in diabetic patients (Jacobson and Weinger, 1998; Gispen and Biessels, 2000; Northam et al., 2006). However, relatively less is known about the slowly developing end-organ damage to the CNS that may present itself by impairment of cognitive functioning. In a perspective population based study of 6370 elderly individuals, patients with diabetes mellitus have an approximate double risk for the development of dementia (Maiese et al., 2007). In animal models with brain/neuronal insulin receptor knockouts, loss of insulin signaling appears to be linked to increased phosphorylation of the microtubule-associated protein tau that occurs during Alzheimer's disease (Maiese et al., 2007). These cerebral complications of both type 1 and type 2 diabetes may be referred to as 'diabetic encephalopathy', a concept introduced several decades ago (Reske-Nielsen et al., 1965). Mijnhout et al. (2006) proposed a new term 'diabetes-associated cognitive decline' (DACD) to facilitate research into this area and to increase recognition of the disorder.

Hyperglycemia leads to production of advanced glycation end products (AGEs), and they damage target cells by three mechanisms. First, AGEs modify the intracellular proteins; hence their function is altered. Second, AGEs modify extracellular matrix components, which interact abnormally with the receptors for matrix proteins (integrins) on cell. Third, plasma proteins modified by AGE precursors bind to AGE receptors (Brownlee, 2001) on endothelial cells, mesangial cells, microglia and macrophages, inducing receptor-mediated production of reactive oxygen species (ROS). This AGE receptor ligation activates transcription factor NFκβ, leading to pro-inflammatory gene expression (Schmidt et al., 1999). It includes expression of cytokines and growth factors by macrophages and mesangial cells (IL-1β, IGF-1, TNF-α, TGF-β, macrophage-colony-stimulating factor, granulocyte-macrophage-colony-stimulating factor and platelet-derived growth factor) and expression of pro-coagulatory and pro-inflammatory molecules by endothelial cells (thrombomodulin, tissue factor and VCAM-1). The

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activation of NF κβ pathway by the hyperglycemia also induces apoptosis in neuronal cells (Mastrocola et al., 2005; Somfai et al., 2006).

Tocochromanols encompass a group of compounds with vitamin E activity essential for human nutrition. Structurally, natural vitamin E includes eight chemically distinct molecules: α-, β-, γ- and δ-tocopherol; and α-, β-, γ- and δ-tocotrienol. Thus, tocotrienols may be viewed as being members of the natural vitamin E family not only structurally but also functionally. Palm oil and rice bran oil represent two major nutritional sources of natural tocotrienol. Taken orally, tocotrienols are bioavailable to all vital organs. Tocotrienols are thought to have more potent antioxidant properties than α-tocopherol (Serbinova et al., 1991; Serbinova and Packer, 1994). The unsaturated side chain of tocotrienol allows for more efficient penetration into tissues that have saturated fatty layers such as the brain and liver (Suzuki et al., 1993). Experimental research examining the antioxidant, free radical scavenging effects of tocopherol and tocotrienols revealed that tocotrienols appear superior due to their better distribution in the fatty layers of the cell membrane (Suzuki et al., 1993; Sen et al., 2007).

With this background, the present study was undertaken to investigate the effects of tocotrienol supplementation on diabetes-induced cognitive impairment and modulation of oxidative–nitrosative stress and cellular death cascade mediators in STZ-induced diabetic rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–280 g), bred in the Central Animal House Facility of Panjab University, Chandigarh (India) were used. The animals were housed under standard laboratory conditions, maintained on a 12 hour light and dark cycle and had free access to food (Hindustan Lever Products, Kolkata, India) and water. The experimental protocols were approved by the Institutional Animal Ethics Committee of the Panjab University, Chandigarh, and conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

2.2. Drugs

Streptozotocin was purchased from Sigma (St. Louis, MO, USA). Tocotrienol (mixture of α-, β-, γ-tocotrienol) was received as a gift sample from Golden-Hope Bioganic, Malaysia Palm Oil Board, Malaysia. A glucose oxidase peroxidase diagnostic enzyme kit was purchased from Span Diagnostic Chemicals, India. TNF-α, IL-1β, caspase 3 ELISA kits (R&D Systems, USA), NF-κB/p65 Active ELISA kit (Imgenex systems) and insulin ELISA kit (DRG, Germany) were purchased. All other chemicals used for biochemical estimations are of analytical grade.

2.3. Induction and assessment of diabetes

A single dose of 45 mg/kg streptozotocin prepared in citrate buffer (pH 4.4, 0.1 M) was injected intraperitoneally to induce diabetes. The age-matched control rats received an equal volume of citrate buffer and used along with diabetic animals. Diabetes was confirmed after 48 h of streptozotocin injection, the blood samples were collected through tail vein and plasma glucose levels were estimated by enzymatic GOD-PAP (glucose oxidase peroxidase) diagnostic kit method. The rats having plasma glucose levels more than 250 mg/dl (Kuhad and Chopra, 2007, 2008) were selected and used for the present study. Streptozotocin (STZ), a β-cytotoxin, increases pancreatic islet O-linked protein glycosylation in a dose-dependent, irreversible fashion and also inhibits GlcNAcase, the enzyme that removes O-GlcNAc from protein. This is the mechanism that accounts for its diabetogenic toxicity (Konrad et al., 2001). Body weight, plasma glucose and insulin levels were measured before and at the end of the experiment to see the effect of tocotrienol on these parameters.

2.3.1. Treatment schedule

Rats were randomly selected and divided in eight groups of 8–10 animals each. First group consisted of non-diabetic control animals, second group was the diabetic control, third, fourth, and fifth groups consisted of diabetic animals treated with tocotrienol (25, 50 and 100 mg/kg/day; p.o., respectively), sixth group comprised of diabetic animals treated with insulin (10 IU/kg/day, s.c.), seventh group consisted of diabetic animal treated with insulin (10 IU/kg/day, s.c.) and tocotrienol (100 mg/kg; p.o.) and seventh group comprised of non-diabetic control animals being administered tocotrienol (100 mg/kg, p.o.) alone. Starting from the third day of experiment till 10th week, the control and diabetic control groups received vehicle of tocotrienol. Tocotrienol was freshly prepared by dissolving in double distilled water after triturating with 5% Tween 80. In the tenth week, animals were tested for learning and memory task in Morris water maze for five consecutive days (Kuhad and Chopra, 2007, 2008). The animals were sacrificed under deep anesthesia, blood was collected by carotid bleeding and serum separated. Brains were rapidly removed and placed on dry ice. First, the cerebral cortex was removed with the help of curved forceps and using a scalpel then hippocampus was punched out with the help of stainless steel punches under inverted microscope (Olympus, USA). A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenates were centrifuged at 200 g for 10 min, at 4 °C and supernatant was used for estimation of lipid peroxidation, superoxide dismutase, catalase and non protein thiols. Cytoplasmic and nuclear fractions were prepared for the quantification of caspase-3 colorimetric detection, total nitric oxide assay, TNF-α ELISA, IL-1β ELISA and NFκβ p56 active ELISA. The samples were stored at −80 °C until processed for biochemical estimations.

2.4. Behavioral assessment

2.4.1. Morris water maze test

Animals were tested in a spatial version of Morris water maze test (Morris et al., 1982; Tuzcu and Baydas, 2006). The apparatus consisted of a circular water tank (180 cm in diameter and 60 cm high). A platform (12.5 cm in diameter and 38 cm high) invisible to the rats, was set inside the tank and filled with water maintained at 28±2 °C at a height of 40 cm. The tank was located in a large room where there were several brightly colored cues external to the maze; these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout the study. The water maze task was carried out for 5 consecutive days after 9th week. The rats received four consecutive daily training trials in the following 5 days, with each trial having a ceiling time of 90 s and a trial interval of approximately 30 s. For each trail, each rat was put into the water at one of four starting positions, the sequence of which being selected randomly. During test trials, rats were placed into the tank at the same starting point, with their heads facing the wall. The rat had to swim until it climbed onto the platform submerged underneath the water. After climbing onto the platform, the animal remained there for 20 s before the commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the rat failed to reach the escape platform within the maximally allowed time of 90 s, it was gently placed on the platform and allowed to remain there for the same amount of time (Kuhad and Chopra, 2007, 2008). The time to reach the platform (latency in seconds) was measured.

2.4.2. Memory consolidation test

A probe trial was performed (Tuzcu and Baydas, 2006; Kuhad and Chopra, 2007, 2008) wherein the extent of memory consolidation was assessed. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. In the probe

trial, the rat was placed into the pool as in the training trial, except that the hidden platform was removed from the pool. The time of crossing the former platform quadrant and the total time of crossing all quadrants were recorded for 1 min.

2.5. Biochemical assessment

2.5.1. Acetylcholinesterase activity

Cholinergic dysfunction was assessed by measuring acetylcholinesterase levels in cerebral cortex and hippocampus according to the method of Ellman et al. (1961). Results were calculated using molar extinction coefficient of chromophore (1.36×10^4 M^{-1} cm^{-1}) and expressed as percentage of control.

2.5.2. Measurement of oxidative stress

The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of Wills (1965). Thiobarbituric acid-reactive substances were quantified using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol of malondialdehyde per mg protein. Tissue protein was estimated using the Biuret method and the brain malondialdehyde content expressed as nanomoles of malondialdehyde per milligram of protein. Non protein thiols were assayed by the method of Jollow et al. (1974). The result was expressed as nmol of NPSH per mg protein. Cytosolic superoxide dismutase activity was assayed by the method of Kono (1978). Catalase activity was assayed by the method of Claiborne (1985).

2.5.3. Estimation of nitrosative stress: total nitric oxide

The quantification of total nitric oxide was done by the help and instructions provided by R&D Systems Total nitric Oxide Assay Kit which involves the conversion of nitrate to nitrite by the enzyme nitrate reductase. The detection of total nitrite is then determined as a colored azo dye product of the Griess Reaction. The Griess reaction is based on the two step diazotization reaction in which acidified NO_2- produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ions. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo derivative which absorbs light at 540570.

2.5.4. Rat Insulin ELISA

Rat Insulin ELISA is a solid phase two-site enzyme immunoassay. Plasma insulin levels were measured by enzyme immunoassay of 25 µl aliquots of plasma with a Rat Insulin ELISA kit (DRG, Germany). During incubation, insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies, which were bound to the plastic surface of the microtitration well. The bound conjugate was detected by reaction with 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped by adding acid to give a colorimetric end point that was read spectrophotometrically.

2.5.5. Rat TNF-alpha and IL-1β ELISA

The quantifications of TNF-alpha and IL-1β were done by the help and instructions provided by R&D Systems Quantikine Rat TNF-alpha and IL-1β immunoassay kit. The Quantikine Rat TNF-alpha and IL-1β immunoassay is a 4.5 hour solid phase ELISA designed to measure rat TNF-alpha and IL-1β levels. The assay employs the sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TNF-alpha or IL-1β has been pre coated in the microplate. Standards, control and samples are pipetted into the wells and any rat TNF-alpha or IL-1β present is bound by the immobilized antibody. After washing away any unbound substance an enzyme linked polyclonal antibody specific for rat TNF-alpha or IL-1β is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of rat TNF-alpha or IL-1β bound in the initial steps. The sample values are then read off the standard curve.

2.5.6. Quantification of NFkappaB p65 unit

The nuclear levels of p65 may correlate positively with the activation of NF-κB pathway. The NF-κB/p65 ActivELISA (Imgenex, San Diego, USA) kit was used to measure NF-κB free p65 in the nuclear lysate. The NF-κB ActivELISA is a sandwich ELISA. Free p65 was captured by anti-p65 antibody coated plates and the amount of bound p65 was detected by adding a second anti-p65 antibody followed by alkaline phosphatase (AKP)-conjugated secondary antibody using colorimetric detection in an ELISA plate reader at 405 nm.

2.5.7. Caspase-3 colorimetric assay

Caspase-3, also known as CPP-32, Yama or Apopain, is an intracellular cysteine protease that exists as a pro-enzyme, becoming activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroanaline (pNA). The cleavage of the peptide by the caspase releases the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate/homogenate is directly proportional to the color reaction. The enzymatic reaction for caspase activity was carried out as using R&D systems caspase-3 colorimetric kit.

2.6. Statistical analysis

Results were expressed as mean±S.E.M. The intergroup variation was measured by one way analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was considered at $P<0.05$. The statistical analysis was done using the SPSS Statistical Software version 14.

Table 1
Effect of tocotrienol and its combination with insulin on body weight, plasma glucose and insulin levels (mean±S.E.M.)

Treatment	Body weight (gm)		Plasma glucose (mg/dl)		Plasma insulin (pmol/l)	
	Onset of study	End of study	Onset of study	End of study	Onset of study	End of study
Control	255±2.21	332±7.84	113±3.96	108±2.19	99±6.53	102±5.51
Diabetes	267±2.13	219±10.86[a]	111±5.62	589±7.48[a]	100±5.45	25±18.45[a]
Diabetes+insulin (10)	252±4.17	312±3.34[b]	110±4.41	156±8.69[b]	99±5.73	65±12.56[b]
Diabetes+Toco (25)	258±5.48	269±5.19[b,c]	103±5.24	362±7.72[b,c]	103±7.43	35±17.44
Diabetes+Toco (50)	256±5.97	289±3.36[b,c]	111±4.61	236±6.62[b,c]	101±4.02	27±12.89
Diabetes+Toco (100)	259±6.93	302±6.82[b,c]	107±5.24	194±8.65[b,c]	96±3.49	32±15.23
Diabetes+insulin (10)+Toco (100)	260±5.33	335±4.8[b,d]	115±3.59	123±5.35[b,d]	99±4.95	59±10.4[b,d]
Toco (100)	261±6.28	341±2.14	115±3.59	114±4.96	105±6.02	92±4.16

[a]($P<0.05$) different from control; [b]($P<0.05$) different from diabetic; [c]($P<0.05$) different from one another; [d]($P<0.05$) different from per se group (0.05). Toco (25) = Tocotrienol, 25 mg/kg, Toco (50) = tocotrienol, 50 mg/kg, Toco (100) = Tocotrienol, 100 mg/kg.

3. Results

3.1. Effect of tocotrienol on body weight, plasma glucose and insulin levels

Ten weeks after streptozotocin injection, plasma glucose levels were highly elevated in diabetic rats (589±7.48 mg/dl) as compared to the control rats (111±5.62 mg/dl). There was a marked decline in the body weights and insulin levels of streptozotocin-treated rats as compared to age matched control rats (Table 1). Chronic tocotrienol treatment (10 weeks) significantly and dose dependently preserved the body weight and plasma glucose levels. The insulin–tocotrienol combination produced marked improvement as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone) [$F(7, 72)=12.121$ ($P<0.05$)]. However insulin levels were not significantly altered in diabetic rats.

3.2. Effect of tocotrienol on diabetes-induced cognitive deficit

The cognitive function was assessed in the Morris water maze test (10th week). The mean escape latency for the trained rats decreased from 70 to 17 s over the course of the 20 learning trials. There was significant difference in transfer latency between diabetic (68±7.04) and control (46±8.60) animals. Chronic tocotrienol treatment significantly decreased mean transfer latency in diabetic animals (Fig. 1A). Diabetic animals showed a lower ability to find the platform and learn its location in the 5th day of training. This poorer performance was improved by the chronic treatment with tocotrienol as evident by decreased latency to find the platform from the 2nd day of training. However, diabetic rats treated with insulin–tocotrienol combination showed much better performance with significant decrease in mean transfer latency as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone) [$F(7,72)=17.921$ ($P<0.05$)].

In the probe trial of the Morris water maze study, this measures how well the animals had learned and consolidated the platform location during the five days of training, animals showed a significant difference (Fig. 1B). The time spent in the target quadrant was significantly lower in diabetic animals as compared to the control group. The rats chronically treated with tocotrienol spent more time in the target quadrant than the diabetic group in the probe test. In addition, diabetic rats treated with insulin–tocotrienol

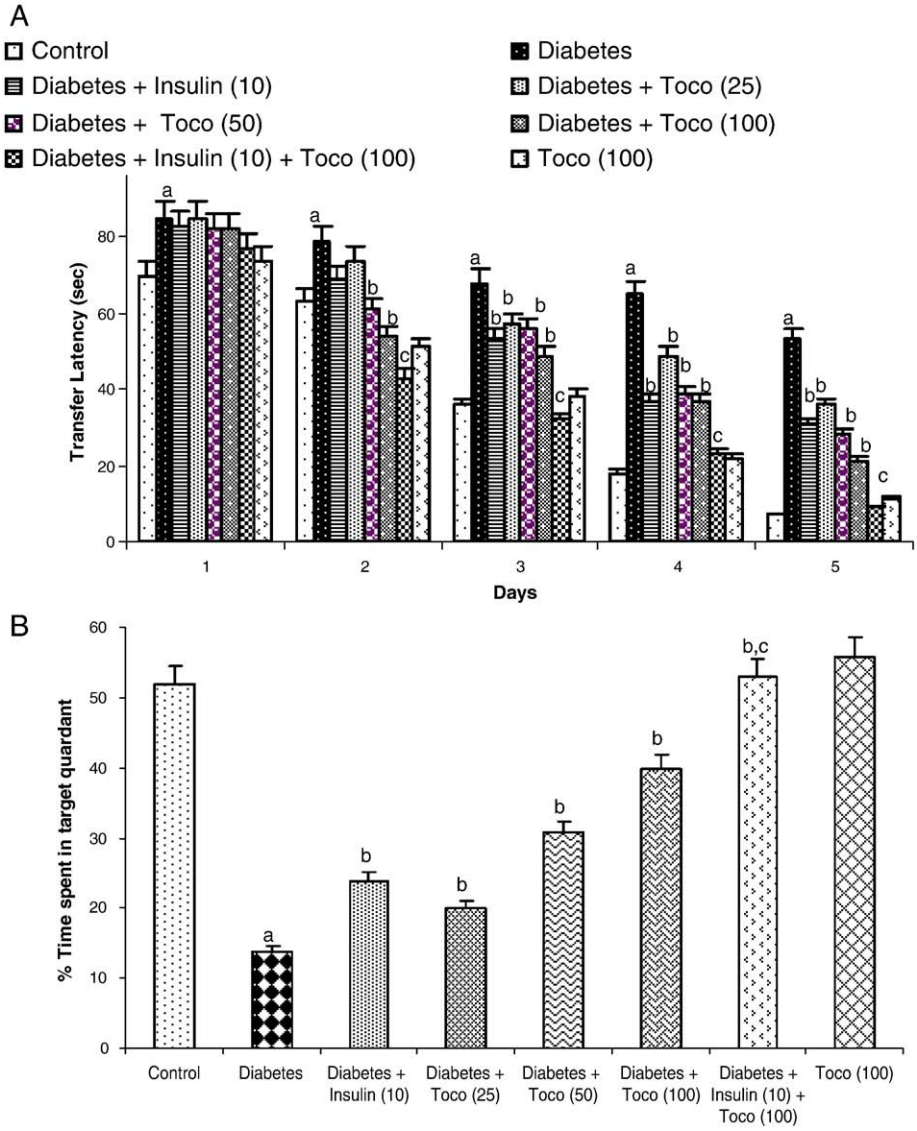

Fig. 1. Effect of tocotrienol (Toco) and its combination with insulin on the performance of spatial memory acquisition phase (mean±S.E.M.) (A) [$F(7,72)=17.921$ ($P<0.05$)] and mean percentage time spent in the target quadrant (percentage control for 10 animals) (B) [$F(7,72)=19.547$ ($P<0.05$)] in control and diabetic rats. a different from control group; b different from diabetic group; c different from per se group. Toco (25) = tocotrienol, 25 mg/kg, Toco (50) = tocotrienol, 50 mg/kg, Toco (100) = tocotrienol, 100 mg/kg.

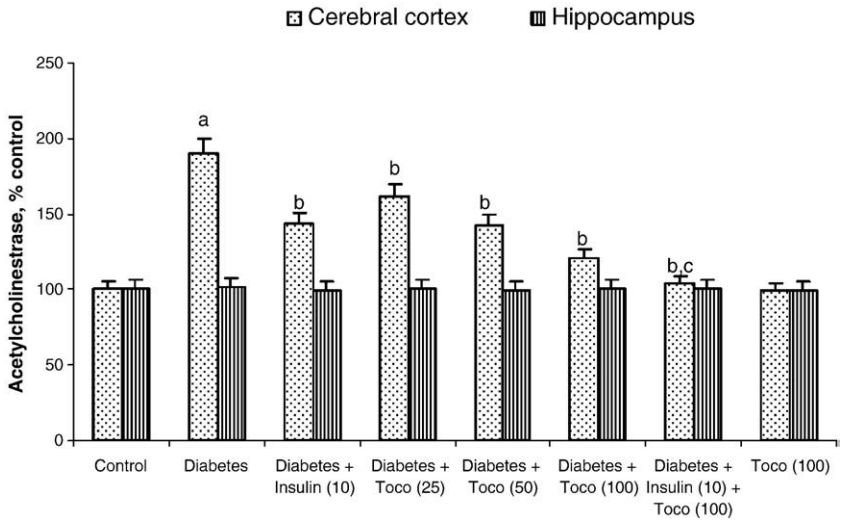

Fig. 2. Effect of tocotrienol (Toco) and its combination with insulin on acetylcholinesterase activity in cerebral cortex and hippocampus of control and diabetic rats [$F(7,72)=414.362$ ($P<0.01$)]. Data are expressed as percentage control for 10 animals. a different from control group; b different from diabetic group; c different from per se group. Toco (25) = tocotrienol, 25 mg/kg, Toco (50) = tocotrienol, 50 mg/kg, Toco (100) = tocotrienol, 100 mg/kg.

combination showed more learning and consolidation as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone) [$F(7,72)=19.547$ ($P<0.05$)].

3.3. Effect of tocotrienol on diabetes-induced changes in acetylcholinesterase activity

Acetylcholinesterase activity was increased by 1.8 fold in cerebral cortex whereas the hippocampal acetylcholinesterase activity did not alter in the diabetic animals after 10 weeks. Chronic tocotrienol treatment significantly and dose dependently prevented this rise in acetylcholinesterase activity, which was further reduced by insulin–tocotrienol combination in the cerebral cortex of STZ-treated rats [$F(7,72)=414.362$ ($P<0.01$)] (Fig. 2).

3.4. Effect of tocotrienol on diabetes-induced changes in lipid peroxidation

Thiobarbituric acid reactive substance levels were increased significantly in the cerebral cortex (2 fold) and hippocampus (2.2 fold) of diabetic rats as compared to control group after 10 weeks (Table 2). Chronic treatment with tocotrienol produced a significant and dose dependent reduction in thiobarbituric acid reactive substance levels in different brain areas of STZ-treated rats. However, diabetic rats treated with insulin–tocotrienol combination significantly prevented this rise in lipid peroxidation as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone) [$F(7,72)=14.325$ ($P<0.05$)].

3.5. Effect of tocotrienol on diabetes-induced changes in the antioxidant profile

The non protein thiols [$F(7,72)=14.393$ ($P<0.05$)] and enzyme activity of superoxide dismutase [$F(7,72)=15.632$ ($P<0.05$)] and catalase [$F(7,72)=16.221$ ($P<0.05$)] significantly decreased in the cerebral cortex and hippocampus of diabetic rats as compared to control group after 10 weeks (Table 2). This reduction was significantly and dose dependently improved by the treatment with tocotrienol in different brain areas of STZ-treated rats. However, diabetic rats treated

Table 2
Effect of tocotrienol and its combination with insulin on lipid peroxide, non protein thiols, superoxide dismutase and catalase levels (mean ± S.E.M.)

Treatment	Brain regions	LPO (nmol/mg protein)	Non protein thiols (mol)	SOD (units/mg protein)	Catalase (K/min)
Control	Cerebral cortex	1.01 ± 0.06	32.53 ± 3.21	8.89 ± 0.38	3.94 ± 0.31
	Hippocampus	1.02 ± 0.10	31.43 ± 2.06	8.64 ± 0.26	3.76 ± 0.39
Diabetes	Cerebral cortex	2.10 ± 0.07[a]	12.87 ± 2.19[a]	4.02 ± 0.28[a]	0.91 ± 0.08[a]
	Hippocampus	2.26 ± 0.09[a]	12.89 ± 3.41[a]	3.94 ± 0.35[a]	0.92 ± 0.06[a]
Diabetes + insulin (10)	Cerebral cortex	1.67 ± 0.11[b]	18.73 ± 1.13[b]	5.99 ± 0.16[b]	1.43 ± 0.10[b]
	Hippocampus	1.81 ± 0.04[b]	17.32 ± 1.16[b]	4.31 ± 1.10[b]	1.36 ± 0.08[b]
Diabetes + Toco (25)	Cerebral cortex	1.94 ± 0.10[b,c]	15.91 ± 1.17[b,c]	5.95 ± 0.11[b,c]	1.26 ± 0.16[b,c]
	Hippocampus	2.05 ± 0.05[b,d]	16.44 ± 1.11[b,d]	4.36 ± 1.17[b,d]	1.19 ± 0.14[b,d]
Diabetes + Toco (50)	Cerebral cortex	1.49 ± 0.16[b,c]	23.03 ± 1.21[b,c]	6.98 ± 0.16[b,c]	2.11 ± 0.15[b,c]
	Hippocampus	1.55 ± 0.11[b,d]	24.89 ± 1.11[b,d]	7.02 ± 1.10[b,d]	1.96 ± 0.05[b,d]
Diabetes + Toco (100)	Cerebral cortex	1.12 ± 0.11[b,c]	29.10 ± 1.08[b,c]	7.81 ± 0.07[b,c]	3.52 ± 0.08[b,c]
	Hippocampus	1.07 ± 0.14[b,d]	29.14 ± 1.10[b,c]	7.99 ± 1.00[b,d]	3.41 ± 0.11[b,d]
Diabetes + insulin (10) + Toco (100)	Cerebral cortex	1.00 ± 0.07[b,e]	34.01 ± 1.02	8.95 ± 0.08[b,e]	3.89 ± 0.14[b,e]
	Hippocampus	1.00 ± 0.02[b,e]	33.23 ± 1.10	8.65 ± 0.04[b,e]	3.73 ± 0.10[b,e]
Toco (100)	Cerebral cortex	1.00 ± 0.10	32.66 ± 1.10	8.81 ± 0.21	4.01 ± 0.17
	Hippocampus	1.01 ± 0.04	32.21 ± 1.03	8.69 ± 0.11	3.95 ± 0.12

[a]($P<0.05$) different from control; [b]($P<0.05$) different from diabetic; [c, d]($P<0.05$) different from one another; [e]($P<0.05$) different from per se group ($P<0.05$). Toco (25) = tocotrienol, 25 mg/kg, Toco (50) = tocotrienol, 50 mg/kg, Toco (100) = tocotrienol, 100 mg/kg.

with insulin–tocotrienol combination significantly restored the endogenous antioxidant profile as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone).

3.6. Effect of tocotrienol on diabetes-induced nitrosative stress

Total nitric oxide levels were significantly elevated in cerebral cortex (2.67 fold) and hippocampus (2.77 fold) of diabetic animals after 10 weeks (Fig. 3). Tocotrienol treatment significantly and dose dependently inhibited this increase in nitric oxide levels, which was further prevented by insulin–tocotrienol combination in different brain areas of STZ-treated rats [$F(7,72)=61.176$ ($P<0.01$)].

3.7. Effect of tocotrienol on tumor necrosis factor-alpha and interleukin-1beta

TNF-α [cerebral cortex (6.2 fold) and hippocampus (6.8 fold) (Fig. 4A)] and IL-1β [cerebral cortex (2.8 fold) and hippocampus (3.2 fold) (Fig. 4B)] levels were significantly elevated in the diabetic animals after 10 weeks. Tocotrienol treatment significantly and dose dependently inhibited TNF-α levels in different brain areas of STZ-treated rats. However, tocotrienol treatment significantly inhibited IL-1β levels in different brain areas of STZ-treated rats, except 25 mg/kg. Moreover, diabetic rats treated with insulin–tocotrienol combination more significantly inhibited TNF-α [$F(7,72)=98.452$ ($P<0.01$)] and IL-1β [$F(7,72)=143.427$ ($P<0.05$)] levels as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone).

3.8. Effect of tocotrienol on nuclear factor kappa beta (NFκβ)

NFκβ p56 subunit was significantly elevated in cerebral cortex (3.8 fold) and hippocampus (4.7 fold) of diabetic animals after 10 weeks (Fig. 4C). Tocotrienol treatment significantly and dose dependently prevented reactive oxygen species induced NFκβ p56 subunit expression in the nuclear fraction of different brain areas of STZ-treated rats, except 25 mg/kg. The levels of NFκβ p56 subunit were further significantly reduced by insulin–tocotrienol combination as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone) [$F(7,72)=112.546$ ($P<0.05$)].

3.9. Effect of tocotrienol on caspase-3

Caspase-3 levels were significantly elevated in cerebral cortex (7 fold) and hippocampus (5.5 fold) of diabetic animals after 10 weeks (Fig. 4D). Tocotrienol treatment (except 25 mg/kg) significantly and dose dependently inhibited apoptosis in different brain areas of STZ-injected rats. However, diabetic rats treated with insulin–tocotrienol combination produced a more pronounced attenuation of caspase 3 expression as compared to their per se groups (diabetic rats treated with insulin and tocotrienol alone) [$F(7,72)=121.231$ ($P<0.05$)].

4. Discussion

In human diabetes, chronic hyperglycemia is associated with a high incidence of progressive dementia (Ryan et al., 2003). The potential mechanisms for this not only include direct effects of hypo- or hyperglycemia and hypo- or hyperinsulimia, but also indirect effects via cerebrovascular alterations (Brands et al., 2004; Lobnig et al., 2005). This study analyzed the role of tocotrienol on the behavioral, biochemical and molecular functions of brains of diabetic rats. STZ-induced diabetes produced marked impairment in cognitive function which was coupled with marked increase in acetylcholinesterase activity in the cerebral cortex. Tocotrienol significantly and dose dependently ameliorated the cognitive impairment in diabetic rats.

Sanchez-Chavez and Salceda (2000) as well as studies from our laboratory (Kuhad and Chopra, 2007) have shown that the activity of acetylcholinesterase was increased in serum and cerebral cortex of diabetic rats but no change was observed in hippocampal acetylcholinesterase activity of hyperglycemic rats. In the present study, chronic treatment with tocotrienol attenuated increase in acetylcholinesterase activity in the cerebral cortex but it did not alter hippocampal acetylcholinesterase activity in diabetic rats.

Direct glucose toxicity in the neurons is especially due to increased intracellular glucose oxidation (Nishikawa et al., 2000), which leads to an increase in reactive species production (Bonnefont-Rousselot, 2002; Evans et al., 2002). In both humans and diabetic rats, oxidative stress seems to play a central role in neuronal damage (Arvanitakis et al., 2004; Kuhad and Chopra, 2007). Recently, it has been reported that oxidative damage to rat synapses contributes to cognitive deficit (Tuzcu and Baydas, 2006). We reported in our previous study that thiobarbituric acid reactive substance levels were significantly increased whereas reduced glutathione, superoxide dismutase and catalase activities were markedly reduced in the cerebral cortex and hippocampus of diabetic rats (Kuhad and Chopra, 2007). In the present study, treatment with tocotrienol returned the levels of brain lipid peroxides, non protein thiols, superoxide dismutase and catalase towards their control values. This observation is corroborated by earlier studies in which tocotrienol exerted a protective effect against oxidative damage in diabetes mellitus

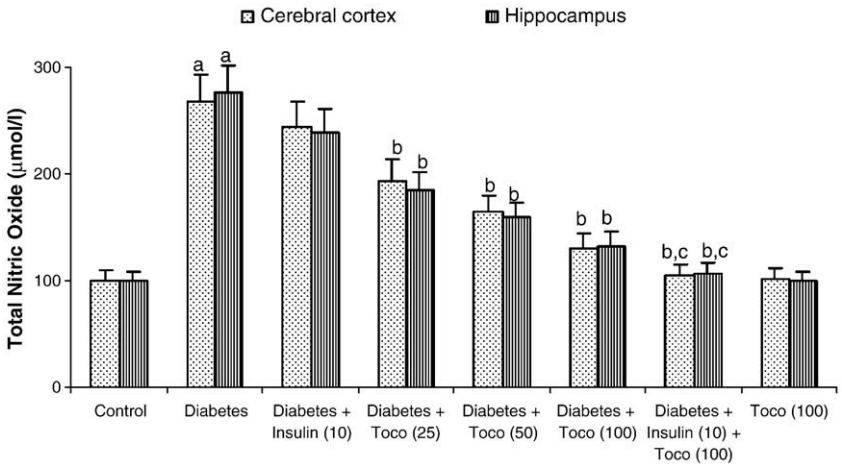

Fig. 3. Effect of tocotrienol (Toco) and its combination with insulin on total nitric oxide in cerebral cortex and hippocampus of diabetic rats [$F(7,72)=14.325$ ($P<0.05$)]. Data are expressed as mean ± S.E.M. a different from control group; b different from diabetic group; c different from per se group. Toco (25) = tocotrienol, 25 mg/kg, Toco (50) = tocotrienol, 50 mg/kg, Toco (100) = tocotrienol, 100 mg/kg.

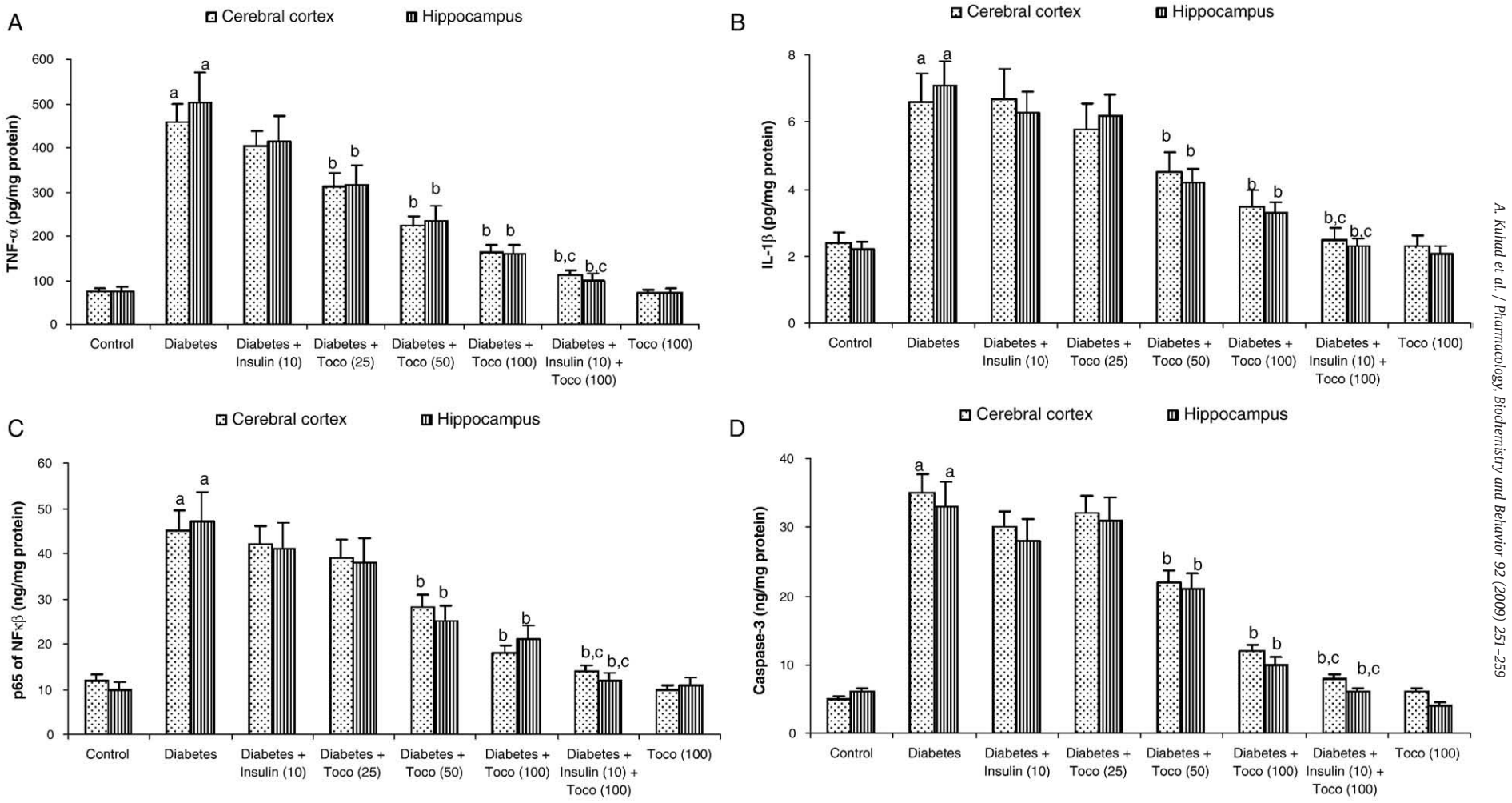

Fig. 4. Effect of tocotrienol (Toco) and its combination with insulin on TNF-α (A), IL-1β (B), p65 subunit of NFκβ (C) and caspase-3 levels in cerebral cortex and hippocampus of diabetic rats. Data are expressed as mean ± S.E.M. a different from control; b different from diabetic group; c different from per se group. Toco (25) = tocotrienol, 25 mg/kg, Toco (50) = tocotrienol, 50 mg/kg, Toco (100) = tocotrienol, 100 mg/kg.

(Kanaya et al., 2004) and protected rat brain mitochondria against oxidative damage (Kamat and Devasagayam, 1995). Tocotrienol is more potent than tocopherol in protecting against free radical-induced impairment of erythrocyte deformability (Begum and Terao, 2002). Topical tocotrienol supplementation inhibits lipid peroxidation in human skin also (Weber et al., 2003).

Besides the enhanced level of reactive oxygen species, NO levels are also increased, and expression of mitochondrial nitric oxide synthase appears to be significantly increased in the brain mitochondria of diabetic rats (Mastrocola et al., 2005). Altered NOS expression may increase peroxynitrite production, which overwhelms the detoxifying reactions so that the effects mediated by NO-derived reactive species prevail. Indeed, peroxynitrite, a harmful oxidant formed by reaction between superoxide and NO, reacts with a variety of molecules, including protein and non-protein-thiols, unsaturated fatty acids and DNA, thus affecting energy conservation mechanisms and oxidative post-translation modification of protein, and ultimately causing neuronal cell death (Murray et al., 2003). Our previous results showed an increase in nitric oxide levels in the cortex and hippocampus of diabetic rats (Kuhad and Chopra, 2007). Chronic treatment with tocotrienol decreased nitric oxide levels in the cytoplasmic lysate. Tocotrienols provided significant protection against the cytotoxicity of a superoxide donor, paraquat, and nitric oxide donors, S-nitrosocysteine and 3-morpholinosydnonimine (Osakada et al., 2004).

Pro-inflammatory cytokines are known to be elevated in several neuropathological states that are associated with learning and memory. Experimental studies reported that the inhibition of long-term potentiation (LTP) in the dentate gyrus region of the rat hippocampus, by tumor necrosis factor (TNF)-alpha, represents a biphasic response, an early phase dependent on p38 mitogen activated protein kinase (MAPK) activation and a later phase, possibly dependent on protein synthesis. TNF-alpha inhibition of LTP is dependent upon the activation of tumor necrosis factor receptor 1 (TNFR1) and mGlu5-receptors along with involvement of ryanodine-sensitive intracellular Ca(2+) stores (Cumiskey et al., 2007). Moreover, in addition to oxidative and nitrosative stress, hyperglycemia is also associated with enhanced inflammatory response (Fukuhara et al., 2007; Kowluru and Kanwar, 2007). Under chronic hyperglycemia, endogenous TNF-α production is accelerated in microvascular and neural tissues, which may cause increased microvascular permeability, hypercoagulability and nerve damage, thus initiating and promoting the development of characteristic lesions of diabetic microangiopathy, polyneuropathy and encephalopathy (Satoh et al., 2003; Brands et al., 2004). Recently, we have reported that uncontrolled diabetes significantly enhanced TNF-α level (Kuhad and Chopra, 2007). A significant inhibition of TNF-α levels by tocotrienol observed in our study is indicative of the fact that tocotrienol contributes to beneficial effects seen in diabetic encephalopathy. Very recently, tocotrienol was also found to abolish tumor necrosis factor alpha (TNF)-induced NFκβ activation (Ahn et al., 2007).

During TNFR1 signaling, NF-κβ is one of the principal signaling pathways, and the activation of IKK requires some of the key adaptor proteins such as TRADD, TRAF2, and RIP (Hayden and Ghosh, 2004; Karin, 1999). One school of thought supports the role for intracellular ROS in TNFα-mediated NF-κβ activation (Hughes et al., 2005; Janssen-Heininger et al., 1999; Piette et al., 1997), corroborated by the inhibitory effect of the mitochondrial-specific antioxidant MitoVitE in human monocytic cell line U937 and human T cell line Jurkat (Hughes et al., 2005). Taken together, the emerging consensus is that ROS, TNF-α and IL-1β are likely to be the general modulators in NF-κβ signaling pathway and caspase-3 activation in different regions of diabetic rat brain (Kim et al., 2007). Further activation of NF-κβ signaling pathway leads to memory disruption and lack of retention in Morris water maze task in the diabetic animals which is positively correlated with increased acetylcholinesterase activity. In the present study, we observed that tocotrienol significantly and dose-dependently suppressed NF-κβ signaling via inhibiting oxidative–nitrosative stress and inflammation. In another study, N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), two antioxidants often used to block NF-κβ activation, suppressed the TNF-mediated NF-κβ pathway via mechanism(s) independent of their antioxidant activity. Moreover, the combination of tocotrienol with insulin produced robust suppression of the TNF-mediated NF-κβ signaling pathway.

Activation of TNF family induces ROS generation via mitochondrial apoptosis inducing factor release (Bajt et al., 2002; Barrett, 2000; Hirata et al., 2001; Lee et al., 2002; Cande et al., 2004). It has already been reported that in type I diabetes, oxidative stress related adaptogenesic pathways are more active in hippocampal neurons than in type II diabetes (Li et al., 2005). To better understand the pathway leading to apoptosis in diabetes, we investigated the expression of apoptosis-related proteins such as caspase-3 in different brain areas of diabetic rats. Caspase-3 represents the predominant caspase in the CNS, both in normal neurodevelopment and in neuropathological states. In the present study, diabetes produced marked increase in caspase-3 activity in cerebral cortex and hippocampus, this effect being amenable to reversal by tocotrienol. A tocotrienol-rich fraction of edible oil derived from palm oil (Tocomin 50%), which contains alpha-tocopherol, and alpha-, gamma- and delta-tocotrienols, significantly inhibited hydrogen peroxide (H2O2)-induced neuronal death in primary neuronal cultures of rat striatum (Osakada et al., 2004). Moreover, tocotrienols have been shown to block oxidative stress-mediated cell death with apoptotic DNA fragmentation caused by an inhibitor of glutathione synthesis, L-buthionine-[S,R]-sulfoximine (Osakada et al., 2004).

The major finding of the study is that insulin alone corrected the hyperglycemia and partially reversed the cognitive decline in diabetic rats. However, combination with tocotrienol not only attenuated the diabetic condition but also reversed cognitive deficits through modulation of TNF-α-induced NF-κβ signaling pathway and caspase-3 in the diabetic rats and thus it may find clinical application to treat neuronal deficit in the diabetic patients.

Conflict of interest

There is no actual or potential conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the work submitted that could inappropriately influence (bias) their work.

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