Original Article

EFFECTS OF STREPTOZOTOCIN INDUCED DIABETES MELLITUS TYPE 1 ON THE RAT BRAIN ANTIOXIDANT STATUS AND ACTIVITY OF ACETYL-CHOLINESTERASE: A NOVEL AND POTENTIAL TREATMENT BY *VITEX NEGUNDO.*

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ABSTRACT

Objective: The objective of the present study was to determine the effect of streptozotocin (STZ) induced diabetes type 1 on activity of acetyl-cholinesterase (AChE) in rat brain and on the brain antioxidant status and also that whether supplementation with the aqueous extract of *Vitex negundo* (AEVN) ameliorates neural degeneration caused by hyperglycemia induced oxidative stress in experimental diabetes.

Methods: Male albino rats weighing 180-200g were made diabetic by the single administration of STZ (45 mg/kg body weight) intraperitoneally. AEVN was administered orally through feeding cannula at a dose of 150 mg/kg body weight daily to STZ-induced diabetic rats for 60 days. On 61st day the rats were sacrificed by cervical dislocation and the effects of the AEVN on fasting blood glucose level. AChE activity and on levels of various oxidant and antioxidant enzyme activity in the brain were appraised.

Results: In the result it is observed that STZ-diabetes caused significant elevation in fasting blood glucose, AChE activity and lipid peroxidase (LPO) level. Whereas activity level of the protective antioxidant enzyme, catalase (CAT), reduced glutathione (GSH) and superoxide dismutase (SOD) exhibited significant decline in STZ-diabetes. Supplementation with AEVN attests significant anti-diabetic and antioxidant potential of it as a prominent decrease in fasting blood glucose level and AChE activity was observed. Similarly, the levels of the protective antioxidant enzymes like SOD, CAT and GSH were increased along with the decrease in the level of oxidant enzyme LPO was observed.

Conclusion: The study emphasizes the involvement of diabetes with neural degeneration and point towards the potential beneficial role of AEVN as an adjuvant therapy to conventional anti-hyperglycemic regimens for the prevention and treatment of diabetic encephalopathy.

Keywords: *Vitex negundo*, Diabetes, Acetyl-cholinesterase, Streptozotocin, Antioxidants.

INTRODUCTION

Diabetes mellitus is a very common and heterogenous metabolic disorder, which is known to cause a variety of complications such as renal failure, blindness, limb amputation, neurological complications, vascular complications of coronary artery disease, cerebrovascular disease and/or premature death [1,2,] affecting 5% of the world's population [3].The World Health Organization (WHO) estimates that more than 300 million people will contract diabetes by the year 2025 and the global cost of treating this disease and its complications could reach US$1 trillion annually [4] and that it is likely to double by 2030, with India, China and United States predicted to have the largest number of affected individuals[5,6].

Diabetes mellitus is known to have potential to cause cerebral injury [7,8]. In diabetic neuropathy, it is known that cerebral uptake of glucose is significantly affected [9-11] and the brain suffers from hypoglycaemic and hyperglycaemic episodes[12].Diabetes-induced hyperglycemia is known to increase the extent of neurological disorders due to inhibition of enzymatic activities connected to neurotransmission [13,14] in the central nervous system (CNS) of mammals and the changes observed have been attributed to alterations occurring in the levels of RNA and proteins [15]. Although different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus, none offers complete glycemic control [16]. Traditional plant medicines are used throughout the world for a range of diabetic presentations. Therefore, an investigation of such agents from traditional medicinal plants has become particularly important. India has a rich history of using various potent herbs and herbal components for treating diabetes. Many Indian plants have previously been investigated for their beneficial use in different types of diabetes [17]. To date, there are many hundreds of herbs and traditional herbal formulas reported to have been used for the treatment of diabetes mellitus [18].Although the ischemic manifestations and cerebral disorders in diabetes have been documented, the pathogenesis of diabetes-induced cerebral dysfunction still remains to be elucidated. *Vitex negundo* Linn. (Family: Verbenaceae) (VN) is commonly known as five leaved Chaste Tree in English and Nirgundi in Hindi [19] VN is large, aromatic, shrub or a small, slender tree with an irregular trunk growing up to 4.5 m in height. Its stem and branches are covered with thin, grey bark, which becomes almost black and scaly when old. It occurs wild in most parts of India near moist places [20]. Although all parts VN are used as medicine in the indigenous system of medicine, the leaves are the most potent for medicinal use [21]. It has been employed in Indian traditional medicinal system for the treatment of various ailments including brain tonic and to improve memory [22,23]. The effectiveness of VN has been scientifically reported for various activities such as anti-inflammatory [24]; gastroprotective [25]; anti-cancer [21]; antioxidant [26,27]; CNS depressant [28]; anticonvulsant [29]; etc. VN leaves contains monoterpenoids iridoids (2-p-hydroxybenzoyl mussaenosidic; nishindaside; negundoside), triterpenoids (betulinic acid; ursolic acid), flavonoids (gardenin A; gardenin B; corymbosin; vitexicarpin; 5-hydroxy-3,6,7,3,4-penta-metoxyflavone; 3,5-dihydroxy-6,7,3,4 tetramethoxyflavanol), phenolic acid (p-hydroxybenzoic acid; 3,4-dihydroxybenzoic acid), and essential oil (sabinene, 4-terpineol, β-caryophyllene, and viridiflorol) [20].

The current study was designed to explore the antidiabetic, neurochemical-antioxidant and cognition protective activities of aqueous extract of *Vitex negundo* (AEVN) leaves in an experimental rat model for Strptozotocin (STZ) induced diabetes. This study may be a holistic approach towards developing a novel drug from Indian folklore for diabetic encephalopathy.

MATERIALS AND METHODS

Animals

Male albino rats of body weight 180–200 g were used and were housed in groups of 3-4 in standard polypropylene cages with wire

mesh top at standard environmental condition of temperature (25±2)°C and relative humidity of 45%-55% under 10 h: 14 h light: dark cycle in the animal house of Department of Bioscience, Barkatullah University, Bhopal. Animals had free access to standard pellet rodent diet and water was provided *ad libitum*. Experiments and maintenance were carried out in accordance with guidelines of the Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA), India and the study was approved by the ethical committee (500/a/CPCSEA/006/2009).

Drugs and chemicals

Streptozotocin [N-(Methylnitrosocarbamoyl)-α-D-glucosamine], (Streptozotocin®, Hi-Media, India); insulin (Human Actrapid®, Novo-Nordisk, India); and other chemicals of analytical grade were procured from the chemical traders and medical shops of Bhopal, India.

Plant material

Commercially available (AEVN) aqueous extract of Vitex negundo (Amruta Herbals Pvt. Ltd.) was dissolved in water (15 mg/100 µl) and was given orally through gavage to rat at a dose of 150 mg/kg bwt.

Induction of diabetes mellitus

The rats were fasted for 12 h (overnight) and diabetes was induced by a single intraperitoneal administration of freshly prepared streptozotocin which were dissolved in 0.1M Na-Citrate buffer of pH 4.5. Prepared STZ at a dose of 45 mg/kg body weight were use for the induction. Diabetes was confirmed after second day of administration by polydipsia, polyuria and by measuring fasting blood glucose concentration, using commercial glucose strip (Accu-Chek® glucometer). Only animals with fasting blood glucose level of 200 mg/dL and above was considered diabetic and used for the experiment.

Experimental design

Twenty four male albino rats were randomly divided into four groups of six each. Group I consisted of control animals; Group II served as diabetic control rats and it received single i. .p. administration of Streptozotocin (45mg/kg bwt.); Group III consisted of Streptozotocin (45 mg/kg bwt., i. .p.) induced diabetic rats and treated with AEVN (150 mg/kg bwt., oral gavage) daily for 60 days; Group IV consisted of Streptozotocin (45 mg/kg bwt. i. .p.) induced diabetic rats and treated with Insulin (4U, i. .m.) Daily for 60 days.

Biochemical estimations of brain

i. Supernatant preparation

On 61st day the fasting blood glucose of animals were checked by Accu-Chek® Active glucometer and sacrificed by cervical dislocation and brain of individual animal was carefully isolated, placed on the petridish, over ice, and weighed. Whole brain samples were rinsed with ice cold normal saline. A 20 mg tissue/ml homogenate of brain samples were prepared by homogenizing in chilled phosphate buffer (pH 7.4). The homogenates were centrifuged at 800 g for 5 minutes at 4°C to separate the nuclear debris. The supernatant thus obtained was centrifuged at 10,500 g for 20 minutes at 4°C to get the supernatant. Supernatant was collected from individual animal from all groups and subjected to the estimation of AChE level, lipid peroxidation (LPO), catalase activity (CAT) and reduced glutathione (GSH).

ii. Estimation of Brain AChE level

Cholinergic dysfunction was assessed by measuring AChE levels in whole brain according to the method of Ellman *et al.* (1961) [30].Briefly, 0.4 ml supernatant was added to a cuvette containing 2.6 ml of sodium phosphate buffer (0.1 M, pH 7.2) to which a 100 µl of Ellaman's reagent (0.5 mM, 19.8 mg DTNB and 0.1 M sodium phosphate, pH 7.2 to make 100 ml) was added and absorbance was measured in spectrophotometer at 412 nm till the increasing absorbance become stable. This stable absorbance was then set to zero and 20 µl of acetylthiocholine iodide (substrate) was added and changes in absorbance per minute were recorded for 10 minutes. The change in absorbance per minutes was calculated. The rate was

calculated by using following formula and AChE activity was measured as µM/l/min/g tissue.

$$R = 5.74 \ (10^{-4}) \times A \ / \ Co$$

Where, R is rate, in moles substrate hydrolyzed per minutes per g of tissue; A is change in absorbance per minutes; Co is the original concentration of tissue i. .e. 20 mg/ml.

iii. Estimation of Brain Lipid Peroxidation (LPO)

Lipid peroxidation was measured by the method of Okhawa *et al.* (1979) [31]. Briefly, to 0.2 ml of test sample, 0.2 ml of SDS, 1.5 ml of acetic acid and 1.5 ml of TBA were added. The mixture was made up to 4 ml with water and then heated in a water bath at 95°C for 60 minutes. After cooling, 1 ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was taken and its absorbance was read at 532 nm. The level of lipid peroxides was expressed as nmoles of MDA released/ mg protein and was calculated using a molar extinction coefficient of 1.56×105 M/cm.

iv. Estimation of Brain Superoxide Dismutase (SOD)

Superoxide dismutase was estimated by the method described by Kakkar *et al.* (1984) [32]. Briefly, 0.5 ml of plasma was diluted to 1.0 ml with ice cold water, followed by 2.5 ml ethanol and 1.5 ml chloroform (chilled reagent). The mixture was shaken for 60 seconds at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate and 0.3 ml of nitro-blue tetrazolium (NBT) and approximately diluted enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of 0.2 ml NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and butanol layer was separated. The colour intensity of the chromogen in the butanol layer was measured in a spectrophotometer at 520 nm. A system devoid of enzyme served as control. One unit of enzyme activity is defined as the enzyme concentration, which gives 50% inhibition of NBT reduction in one minute under assay conditions. SOD activity was expressed as U/mg of protein.

v. Estimation of Brain Reduced Glutathione (GSH)

Reduced glutathione (GSH) is estimated by methods based on principles of methods of Moron *et al.* (1979) [33]. 0.5 ml of supernatant was taken in a test tube and 2 ml of distilled water was added, mixed well. Then it was centrifuged for 5 min at 5000 rpm. 0.5 ml of supernatant was taken, to which 0.5 ml of TCA (5%) was added and then centrifuged for 10 min at 10,000 rpm. 0.5 ml of supernatant was taken to which 2.5 ml of phosphate buffer (pH 8) was added. To this 1 ml DTNB was added. This solution was inverted for 3 times to mix. The absorbance was read on spectrophotometer at 412 nm within 4 min. of preparing the mixtures. Standard graph of reduced glutathione GSH concentrations was plotted. Determination of reduced glutathione GSH concentration was done from the graph and expressed as µg/mg protein.

vi. Estimation of Brain Catalase (CAT)

Catalase activity was measured by the method of Aebi (1979) [34]. 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 ml of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as µmoles of H_2O_2

Statistical Analysis

Results were expressed as mean ± S. .E. .M. The inter group 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 Sigma Stat Statistical Software version 3.5.

RESULTS

After induction of diabetes on the 61st day, the fasting blood glucose level was highly elevated in the diabetic animals (P < 0.001). After treatment with AEVN leaves the fasting blood glucose level was significantly decreased compared to that of the diabetic controls (P<0.001). And (4U/day) insulin significantly decreased the serum glucose level (P<0.001) (Table 1).

Table 1: Effect of AEVN Leaves on Fasting Blood Glucose Level Of STZ-Diabetic Rats.

Groups	n	Fasting Blood Glucose (mg/dl)	
		Initial (1st day)	Final (61st day)
Control	6	82.0±1.7	82.0±1.9
Diabetic Control	6	218.0±1.8***	325.0±2.3***
Diabetic+AEVN	6	218.0±1.3***	98.0±1.0NS,ΔΔΔ
Diabetic+Insulin	6	221.0±1.8***	85.0±1.8NS,ΔΔΔ

± = Standard Error of Mean (SEM) of 6 Animals. NS = Non Significant, *=P< 0.05, **=P<0.01, ***=P<0.001 *vs* Control group, ᐃ=P < 0.05, ᐃᐃ=P<0.01, ᐃᐃᐃ=P<0.001 *vs* Diabetic control group.

Acetylcholinesterase activity was significantly increased in diabetic controls when compared with the normal controls (P<0.001). After treatment with AEVN leaves showed a significant decrease in acetylcholinesterase activity in diabetic animals (P<0.001), and 200 mg/kg of piracetam-treated group showed a significant decrease in acetylcholinesterase activity (P<0.01), whereas (4U/day) insulin-treated group did not show the significant effect in this regard, (Table 2).

Table 2: Effect of AEVN Leaves on AChE Level in Brain of STZ-Diabetic rats

Groups	n	AChE activity (µM/min/l/g tissue)
Control	6	3.67±0.47
Diabetic Control	6	6.36±0.30***
Diabetic+AEVN	6	4.07±0.42NS,ΔΔΔ
Diabetic+Insulin	6	6.17±0.24***

± = Standard Error of Mean (SEM) of 6 Animals. NS = Non Significant, *=P 0.05, **=P<0.01, ***=P<0.001 *vs* Control group, ᐃ=P < 0.05, ᐃᐃ=P<0.01, ᐃᐃᐃ=P<0.001 *vs* Diabetic control group.

The results showed a significant increase in LPO level (P<0.01), and significant decreases in SOD (P<0.01), CAT (P<0.001) and Reduced GSH (P<0.01) levels in the diabetic controls compared to those of the normal controls. After treatment with AEVN leaves STZ-diabetic rats showed a significant decrease in LPO level and improvement in SOD,CAT and reduced GSH levels (P<0.001). After treatment with (4U/day) insulin, it showed a significant reduction (P<0.001) in LPO level while a significant increase in SOD level in the diabetic animals (P<0.001) (Table 3).

Table 3: Effect of AEVN Leaves on LPO, SOD, CAT and Reduced GSH Levels in Brain of STZ-Diabetic Rats.

Groups	n	LPO (MDA nmol/mg protein)	SOD (U/mg protein)	Reduced GSH (µg/mg protein)	CAT (µmoles of H_2O_2 utilised / second)
Control	6	1.49±0.02	7.75±0.38	22.24±0.7	3.12±0.29
Diabetic Control	6	3.65±0.02**	5.17±0.27**	17.13±0.84**	0.86±0.05***
Diabetic+AEVN	6	1.58±0.01NS,ΔΔΔ	6.90±0.22NS,ΔΔΔ	20.67±0.78NS,ΔΔΔ	2.75±0.20NS,ΔΔΔ
Diabetic+Insulin	6	1.55±0.01NS,ΔΔΔ	7.51±0.56NS,ΔΔΔ	20.01±0.90NS,ΔΔΔ	1.98±0.11*,ΔΔΔ

± = Standard Error of Mean (SEM) of 6 Animals. NS = Non Significant, *=P< 0.05, **=P<0.01, ***=P<0.001 *vs* Control group, ᐃ=P< 0.05, ᐃᐃ=P<0.01, ᐃᐃᐃ=P<0.001 *vs* Diabetic control group.

DISCUSSION

This research work is one of a series of studies showing that chronic hyperglycemia causes an imbalance in the oxidative status of the nervous tissue and that the resulting free radicals damage the brain through a peroxidative mechanism. The STZ diabetic rat serves as an excellent model to study the molecular, cellular and morphological changes in the brain induced by stress during diabetes[35]. Under normal conditions, the generation of free radicals or of active species in the brain, as in other tissues, is maintained at extremely low levels[36]. Diabetes also contributes to cerebrovascular complications, reductions in cerebral blood flow, disruption of the blood brain barrier and cerebral edema [37]. All of these neurochemical and neurophysiological changes ultimately contribute to the long-term complications associated with diabetes, including morphological abnormalities, cognitive impairments and increased vulnerability to pathophysiological event [38]. Currently available drugs for the treatment of Diabetes mellitus have a number of limitations, such as adverse effects and a high rate of secondary failure [39].As there is a growing trend towards using natural remedies adjunct to conventional therapy, traditionally used plants might provide a useful source of new hypoglycemic compounds [40]. A number of plants have been reported to possess hypoglycemic effects and the possible mechanism suggested for such hypoglycemic actions could be through the increased insulin secretion from β-cells of islets of Langerhans or its release from bound insulin. Alternatively such hypoglycemic effects of plant extracts could also be because of their insulin-like actions [41,42] Similar mechanisms may be considered responsible for the hypoglycemic action shown by AEVN in diabetic rats.

In diabetic condition, a significant rise in acetylcholinesterase activity in the brain of the diabetic animals is observed, as this enzyme hydrolyses acetylcholine present in the brain and results in cognitive decline [43]. AChE enzyme is essential in maintaining the normal function of the nervous system, since it rapidly terminates the action of ACh released into the synapse. Cholinergic interneurons in the striatum are even richer source of AChE [44]. One of the treatment strategies to enhance cholinergic functions is the use of AChE inhibitors that increase the availability of ACh in central cholinergic synapses [44,45]. Sixty days treatment with AEVN leaves attenuated the increase in acetylcholinesterase activity in the brain of the diabetic animals. This decrease in acetylcholinesterase activity is may be due to flavonoids content of the extract [46].

Lipid peroxidation is one of the characteristic features of chronic diabetes and lipid peroxidation mediated tissue damage has been observed in diabetic conditions [47]. Hyperglycemia generates reactive oxygen species (ROS), which in turn cause lipid peroxidation and membrane damage [48]. The increased lipid peroxidation during diabetes, as found in the present study may be due to the inefficient anti-oxidant system prevalent in diabetes. The elevated lipid peroxidation is responsible for the formation of lipid hydroperoxides in membrane and would result in damage of the membrane structure and inactivation of membrane- bound enzymes. The accumulation of lipid peroxides adds hydrophilic moieties into the hydrophobic phase and thereby brings about changes in the membrane permeability and cell functions [49]. Diabetes-induced oxidative damage [50] is responsible for the changes occurring in activities of membrane-bound enzymes of significance in neuronal

activity. Earlier studies have also reported an increase in lipid peroxidation leading to impaired neuronal activity in diabetes mellitus [51]. Treatment with AEVN leaves significantly lowered LPO in the brain of diabetic rats and simulated the anti-diabetic response of insulin treatments.

Glutathione (GSH), a tripeptide normally present in high concentrations intracellularly constitutes the major reducing capacity of the cytoplasm [52] and protects the cellular system against the toxic effects of lipid peroxidation [53].The diabetic animals in the present study registered lowered levels of GSH reflecting its increased utilization owing to oxidative stress while, a significant elevation of GSH levels in AEVN leaves administered to diabetic rats coincided with a significant decline in lipid peroxidation. It appears that the effect of AEVN leaves on GSH could be at two levels – either through increasing the biosynthesis of GSH or by inhibiting its utilization by reducing oxidative stress.

The antioxidant enzymes SOD and CAT play an important role in reducing cellular stress. SOD scavenges the superoxide radical by converting it to hydrogen peroxide and molecular oxygen [54], while CAT brings about the reduction of hydrogen peroxides and protects higher tissues from the highly reactive hydroxyl radicals [55]. In the present investigation both these enzymes registered low levels of activity in diabetic controls indicating diabetes-induced stress. Such a decline in these enzyme activities has also been reported earlier [56,57]. The AEVN leaves when administered to the diabetic animals improved both SOD and CAT activities substantially, reflecting the antioxidant potency of VN. The effects of AEVN leaves on antioxidants (GSH, SOD, CAT and LPO) were found to be better than those of insulin administered diabetic animals. Our data confirmed that type 1 diabetes is an oxidative state that causes a statistically significant decrease of the rat brain total antioxidant status.

Hyperglycemia is known to induce a disruption of the mitochondrial membrane potential and an increase in the formation of reactive oxygen species (ROS) [58]. This diabetes-induced oxidative stress has been demonstrated to play an important role in the occurring brain damage due to diabetes, in both humans and animals suffering from the disease [59]. This damage is mainly caused by: increased ROS formation, increased malondialdehyde levels, as well as decreased catalase and superoxide dismutase activities [60,61]. Our data reveal that AEVN leaves administration can be a protective treatment against the diabetes-induced oxidative stress occurring in the rat brain, since it can efficiently reverse the total antioxidant status of diabetic rat brain into those of the control group.

CONCLUSION

In conclusion, diabetes-induced hyperglycaemic injury accounts for the oxidative damage and down-regulation of the activities of antioxidant and membrane-bound enzymes of functional significance leading to impaired neuronal function. Our study shows that AEVN leaves intake is effective in up-regulating the antioxidant defence mechanism by attenuating the oxidative stress. Changes in the cholinergic system, suggest that AEVN leaves have neuroprotective effect in STZ-induced diabetes. Thus, treatment with AEVN leaves for ameliorating the cognitive decline, cholinergic dysfunction, and oxidative stress in the diabetic animals can innovate the clinical application in treating neuronal deficit in the diabetic patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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