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Sodium Orthovanadate and *Trigonella Foenum Graecum* Prevents Neuronal Parameters Decline and Impaired Glucose Homeostasis in Alloxan Diabetic Rats

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Abstract: Hyperglycemia is the most important contributor in the onset and progress of diabetic complications mainly by producing oxidative stress. The present study was carried out to observe, the antihyperglycemic effect of sodium orthovanadate (SOV) and *Trigonella foenum graecum* seed powder (TSP) administration on blood glucose and insulin levels, membrane linked enzymes (monoamine oxidase, acetylcholinesterase, Ca^{2+}ATPase), intracellular calcium (Ca^{2+}) levels, lipid peroxidation, membrane fluidity and neurolipofuscin accumulation in brain of the alloxan induced diabetic rats and to see whether the treatment with SOV and TSP was capable of reversing the diabetic effects. Diabetes was induced by administration of alloxan monohydrate (15 mg/100 g body weight) and rats were treated with 2 IU insulin, 0.6 mg/ml SOV, 5% TSP in the diet and a combination of 0.2 mg/ml SOV and 5% TSP separately for three weeks. Diabetic rats showed hyperglycemia with almost four fold high blood glucose levels. Activities of acetylcholinesterase and Ca^{2+}ATPase decreased in diabetic rat brain. Diabetic rats exhibited an increased level of intracellular Ca^{2+} levels, lipid peroxidation,

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neurolipofuscin accumulations and monoamine oxidase activity. Treatment of diabetic rats with insulin, TSP, SOV and a combined therapy of lower dose of SOV with TSP revived normoglycemia and restored the altered level of membrane bound enzymes, lipid peroxidation and neurolipofuscin accumulation. Our results showed that lower doses of SOV (0.2 mg/ml) could be used in combination with TSP in normalization of altered metabolic parameters and membrane linked enzymes without any harmful side effect.

Introduction

Diabetes is characterized by hyperglycemia and metabolic abnormalities resulting from decreased insulin levels causing metabolic and other physiological changes in various organs including brain (Mohammad et al., 2006; Baquer et al., 2009, 2011; Kumar et al., 2011, 2012a, b). Hyperglycemia is the most important contributor in the onset and progress of diabetic complications mainly by producing oxidative stress. High oxidative stress can lead to microvascular cerebral diseases, e.g. cerebral hemorrhage, stroke and neurodegenerative disorders (Pitocco et al., 2013; Tiwari et al., 2013).

Many antidiabetic compounds like vanadium, a trace metal and plant extracts have been explored as an alternative to insulin therapy for over a decade in various diabetic models (Baquer et al., 2011). Vanadium salts such as sodium orthovanadate mimic several of the metabolic and growth promoting effects of insulin (Mishra et al., 2010; Kumar et al., 2012c). Vanadium is considered as a potential antidiabetic element but it is highly toxic when given in excessive dose to animals and humans (Ghareeb and Hussen, 2008).

Trigonella foenum-graecum Linn. is an annual herb belonging to the family *Leguminosae*, widely grown in India, Egypt, and Middle Eastern countries (Srinivasan, 2006; Baquer et al., 2011). The chemical constituents of *Trigonella* seed include volatile oils, alkaloids, saponins, sapogenins, flavonoids and mucilage. 4-hydroxyisoleucine, an amino acid extracted and purified from *Trigonella* seed displays antihyperglycemic and insulinotropic property *in vitro* and *in vivo* in type 2 diabetes mellitus rat model (Fuller and Stephens, 2015). Petit et al. (1995) reported the isolation of furostanol saponins called trigoneoside Ia, Ib, IIa, IIb, IIIa, IIIb; glycoside and trifoenoside A in *Trigonella* seeds. Various reports have demonstrated and summarized that the *Trigonella* seeds have hypoglycemic, hypocholesterolemic and hyperinsulinomic effects on type 1 and type 2 diabetes mellitus patients and experimental diabetic animals (Basch et al., 2003; Srinivasan, 2006; Kumar et al., 2012a, b, c; Yadav and Baquer, 2014; Fuller and Stephens, 2015).

Oxidative stress and free radicals generation during diabetes leads to damage various membranes bound enzymes, membrane fluidity and lipid peroxidation (Kumar et al., 2012c). Monoamine oxidase (MAO), a mitochondria bound enzymes plays an essential role in the turnover of monoamine neurotransmitters (Soto-Otero et al., 2001). Uncontrolled diabetes is associated with a significant

disturbance of brain monoamine metabolism (Gupta et al., 1992). Ca^{2+}ATPase plays an important role in maintaining homeostatic of calcium levels in brain (Doğru et al., 2005). Diabetes-induced hyperglycemia is known to increase the extent of neurological disorders due to inhibition of AChE (acetylcholinesterase) activity, connected to neurotransmission (Ghareeb and Hussen, 2008; Ahmed and Tarannum, 2009). Membrane fluidity is an effective index of diabetic complications. It has also been reported that free radicals generated during diabetes deteriorate membrane structure and decreases membrane fluidity. Lipid peroxidation and membrane fluidity have been implicated in neurodegenerative disease process and diabetic complications (Hong et al., 2004, Baquer et al., 2011).

Previous studies from our laboratory have demonstrated that lower doses of SOV (sodium orthovanadate) in combination with TSP (*Trigonella foenum graecum* seed powder) restore altered carbohydrate metabolism and antioxidant status in diabetic rats (Mohammad et al., 2006; Baquer et al., 2009, 2011; Kumar et al., 2012c; Yadav and Baquer, 2014).

The aim of the present study was to investigate the anti-diabetic potential of low doses of SOV (0.2 mg/ml) in combination with TSP on membrane linked enzymes, intracellular Ca^{2+} levels, lipid peroxidation, membrane fluidity and neurolipofuscin accumulation in brain of the alloxan diabetic rats.

Material and Methods

Animals

Adult female albino rats of the Wistar strain, weighing between 180–220 g were used for all the experiments. Animals were kept in the animal house maintained at temperatures of 22–26 °C and relative humidity of 55% with a 12 h each of dark and light cycle. The animals were fed standard chow (Hindustan Lever Ltd., India) and given tap water *ad libitum* for the time of treatment before sacrifice. All the animal procedures were approved by the Institutional Animal Ethical Committee (IAEC) of Jawaharlal Nehru University, New Delhi, India.

Methodology

Overnight-starved rats (duration of starvation 10–12 h) were made diabetic by a single subcutaneous injection of alloxan monohydrate (15 mg/100 g body weight) freshly prepared in 0.154 M sodium acetate buffer (pH 4.5) (vehicle) according to the method of Mohammad et al. (2006). Control animals were given only the vehicle. The alloxan induced diabetic rats were injected with 2 IU of protamine-zinc insulin for the next 7 days; this procedure decreases the mortality of the diabetic animals. Animals were used three weeks after stopping insulin. The severity of diabetes was checked in rats by using urine glucose strips (Diastix, Bayer Diagnostic, India). Animals (n=6) were then grouped into control (C), diabetic (D), diabetic treated with insulin (D+I), diabetic treated with TSP (D+T), diabetic treated with SOV (D+V), and diabetic treated with both TSP and SOV (D+T+V).

Protamine zinc insulin (2 IU) suspension was administered intraperitoneally to diabetic animals (D+I) every day for three weeks. The diabetic treated with TSP (D+T) were given 5% finely powdered seeds of *Trigonella foenum graecum* (AGMARK BRAND, purchased from local market) in powered rat feed (i.e. 5 g of dry TSP in 95 g of powdered rat feed) for three weeks. The plant material was identified as per the literature of Ayurveda and by local experts of herbal gardens and further taxonomically validated by Dr. G. P. Rao, Department of Botany, Sri Venkateswara College, University of Delhi, New Delhi. The most effective dose of 5% TSP in the diet was in previous studies (Mohammad et al., 2006; Kumar et al., 2012b, c).

SOV was given at a dose of 0.6 mg/ml in drinking water (freshly prepared) consecutively for three weeks to the diabetic animals (D+V), SOV was dissolved in drinking water with 0.5% sodium chloride to reduce its toxicity (Mohammad et al., 2006). The diabetic rats treated with TSP and SOV (D+T+V) were given 0.2 mg/ml of SOV dissolved in tap water containing 0.5% sodium chloride. The method had been used earlier (Mohammad et al., 2006; Kumar et al., 2012a, c).

Preparation of homogenates and subcellular fractions

Animals were sacrificed by cervical dislocation. Whole brains were rapidly excised, and washed with chilled normal saline. Tissue homogenates (1:10) were prepared in homogenizing buffer containing 0.25 M sucrose 0.02 M triethanolamine (pH 7.4) and 0.12 mM dithiothreitol. The pellet obtained after centrifugation at 12,000 rpm using SM 22 rotor on a high speed cooling centrifuge (SORVALL 5CA) containing crude synaptosomes (mitochondria and synaptosomes). The supernatant fraction was separated from the pellet and was used as the soluble fraction (Siddiqui et al., 2005; Kumar et al., 2012b).

Biochemical assays

Assay of MAO (EC: 1.4.3.4)

MAO activity was measured in the synaptosomal fractions of control and diabetes treated animals according to the method of Catravas et al. (1977) as modified by Mayanil et al. (1982) the activity was measured as amount of the reaction product, 4-hydroquinoline and was determined spectrophotometrically at 330 nm. One unit of enzyme is defined as one μmole of 4-hydroxyquinoline produced per mg protein per minute at 37 °C.

Assay of Ca^{2+}ATPase (EC: 3.6.3.8)

Ca^{2+}ATPase activity was measured in the synaptosomes using a colorimetric method described by Desaiah et al. (1985) and as used by Kumar et al. (2012a). Ca^{+2}ATPase enzyme activity was calculated by the difference of the ATP hydrolysis in the presence and absence of (0.2 mM EGTA) $CaCl_2$. The specific activity of the enzyme is expressed as μmole Pi released/mg protein/min.

Assay of AChE (EC: 3.1.1.7)
The assay of AChE in the synaptosomes according to the method described by Mantha et al. (2006) the activity was measured as amount of the reaction product, thiocholine and was determined spectrophotometrically at 412 nm. The specific activity of the enzyme is expressed as μmoles of thiocholine produced per min per gram of protein (μmoles/min/protein) at room temperature.

Measurement of lipid peroxidation
A secondary product of lipid peroxidation, 4-hydroxynonenal (4-HNE), was measured spectrofluorimetrically in brain homogenates essentially by the method of Tappel et al. (1973). Quinine sulphate (0.1 μg/ml) in 0.05 M H_2SO_4 was used as a standard. The amount of 4-HNE was expressed as percentage fluorescence of control from 1 ml of 10% homogenate (w/v).

Membrane fluidity
The synaptosomal fractions were labelled with 1,6-diphenyl-1,3,5-hexatriene, a fluorescent probe by incubating equal volume of a membrane suspension containing 100 μg/ml of protein in phosphate buffer and 2 μM 1,6-diphenyl-1,3,5-hexatriene suspension in the same buffer. Excitation and emission wavelengths were, respectively, 365 and 428 nm. Polarization (P) measurements were carried out on a model SLM 4800 polarization spectroflourometer as described by Mantha et al. (2006).

Measurement of intrasynaptosomal calcium levels
Intrasynaptosomal calcium ion concentration was determined by the dual wavelength method described by Grynkiewicz et al. (1985) using calcium sensitive fluorescent probe Fura-2AM. Fluorescence intensity of Fura-2 in crude synaptosomes was measured using Cary Eclipse Spectrofluorimeter (Varian, Palo Alto, USA) with the filters set at 510 nm for emission and 340/380 nm for excitation. Intracellular calcium was estimated using the relation described.

$$[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times Sf_2/Sb_2$$

Where: R, R_{max} and R_{min} represented the ratio of fluorescence (F340/F380) of the sample, maximum dye response in presence of saturating concentration of calcium and minimal dye response in presence of excess ethyleneglycol-bis-(β aminoethyl ether)-N,N'-tetra acetic acid (EGTA), respectively; K_d is the dissociation constant, and Sf_2 and Sb_2 denote the fluorescence of fura-2AM at zero calcium and full calcium saturation, respectively, at the excitation wavelength of 380 nm.

Histochemical localization and distribution of neurolipofuscin
Intraneuronal lipofuscin accumulation in the cerebral hemispheres of the control and diabetic treated rats were observed in 5 micron thick paraffin embedded, deparaffinised sections according to the method of Riga and Riga (1974) and

used by Kumar et al. (2012a) by fluorescence microscopy using a Zeiss Orthomat microscope equipped with fluorescence attachments with Ploemipak Epi illuminator, H2 cube (wide band), and exciter filter 390–490 nm was used.

Insulin levels
Serum insulin levels were measured by an enzyme immunoassay using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden).

Blood glucose
Blood glucose was estimated by Glucose Enzokit from Ranbaxy Laboratories India, using glucose oxidase method.

Protein estimation
Protein was estimated in brain subcellular fractions by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Statistical analysis
Results were analysed by means of Prism 5.0 (GraphPad, San Diego, CA, USA). All data were calculated as means ± SEM of 4–6 separate values. The ANOVA test followed by Dunnet Multiple Comparison test was employed for statistical comparison between control and various experimental groups. Values with $p<0.05$ were considered as statistically significant.

Chemicals
All purified enzymes, coenzymes, substrates, standards and buffers were from Sigma Chemicals Company, USA. All other chemicals were of analytical grade and were from SRL and Qualigens, India.

Results

Effect of antidiabetic compounds on general parameters
Animals with serum glucose levels, more than 350 mg/dl were taken for the diabetic group. These animals had glycosuria, polydipsia, polyphagia and a reduced rate of development. Physiological parameters like body weight, tissue weight, protein levels, insulin levels and blood glucose levels as observed in controls and all the experimental groups and results are presented in Table 1.

Changes in body and brain weights
The body weights of diabetic rats as compared with the controls were significantly reduced ($p<0.001$) after three weeks of experiments. Treatment with insulin (D+I) for three weeks effectively improved the weight gain of the diabetic rat. TSP given alone in the diet and TSP and SOV (D+T+V) given in combination resulted in significant increase in body weights when compared with the diabetic

Table 1 – Changes in body weight, protein content, glucose and insulin levels of the control (C), diabetic (D), and diabetic rats treated with insulin (D+I), SOV (D+V), TSP (D+T) and combined dose of SOV and TSP (D+T+V)

General parameters	C	D	D+I	D+V	D+T	D+T+V
Body weight (g)	220 ± 16.7	140 ± 18.5[a]	205 ± 16.3	165 ± 12.4	200 ± 14.6	205 ± 12.9
Blood glucose (mg/dl)	86 ± 6.2	440 ± 15.5[a]	102 ± 5.3	111 ± 7.2	125 ± 9.8[b]	105 ± 7.2
Protein content (mg/g) Brain mitochondria	54.5 ± 3.17	57.3 ± 4.62	55.3 ± 5.43	56 ± 4.71	56.5 ± 3.95	55.5 ± 2.77
Serum insulin (mU/ml)	0.013 ± 0.004	0.002 ± 0.0002[c]	0.034 ± 0.005[b]	0.0068 ± 0.0004	0.007 ± 0.0004	0.008 ± 0.0002

Each value is a mean ± SEM of six to eight separate values from two to three experiments. The comparisons of experimental values are with the control values. Statistical significance: [a]p<0.001, [b]p<0.01, [c]p<0.05. The protein content is in the supernatant fractions of all tissue. In D+V, SOV concentration was 0.6 mg/ml, and D+V+T, SOV concentration was 0.2 mg/ml

rats. The brain weight, when calculated per 100 g body weight, of the control and experimental groups did not show a statistical difference.

Blood glucose levels

Diabetic rats showed a fivefold ($p<0.001$) increase in blood glucose concentration when compared with control rats. The high glucose levels were normalized in all diabetic animals with the antidiabetic treatments. Diabetic animals treated with SOV (D+V) and insulin (D+I) groups showed the best reversal. Three weeks of treatment with TSP (D+T) separately resulted in a significant ($p<0.01$) reduction in hyperglycemia in the diabetic rats. Combined dose of SOV and TSP (D+T+V) was more effective than SOV (D+V) alone and TSP (D+T) alone in lowering hyperglycemia in the diabetic rats.

Insulin levels

There was a significant ($p<0.05$) decrease of almost 80% in insulin levels in the diabetic group when compared with the control. Treatment of diabetic rats with SOV, TSP and TSP and SOV in combination dose restored the insulin levels to 3.4-fold (D+V), 3.5-fold (D+T) and 4-fold (D+T+V), respectively, of the diabetic levels. The combined dose in (D+V+T) group was the most effective of all the treatments used.

Changes in protein content

The protein contents of brain fractions were not significantly affected by diabetes and various antidiabetic treatments.

Effects of antidiabetic compounds on membrane bound enzymes

MAO activity

The changes in the activity of MAO in synaptosomes of control, diabetic and diabetic rats after three weeks of treatment with antidiabetic compounds, were measured and results are shown in Figure 1. The MAO activity was increased by 71% ($p<0.01$) in diabetic rats, when compared with control rats. The activity of MAO of diabetic animals were decrease in D+T, D+V, and D+T+V group of rats when compared to untreated diabetic rats. There was a significant changes in D+T group ($p<0.05$) with respect to control group. The combined treatment of MAO activity with TSP and SOV (D+T+V) for three weeks was found to be the most effective treatment for restoration.

Ca^{2+}ATPase activity

The activity of Ca^{2+}ATPase in synaptosomes of diabetic animals were decrease in D+T, D+V, and D+T+V group of rats when compared to untreated diabetic rats. After three weeks, a decrease of 48% ($p<0.01$) was observed in the activity of

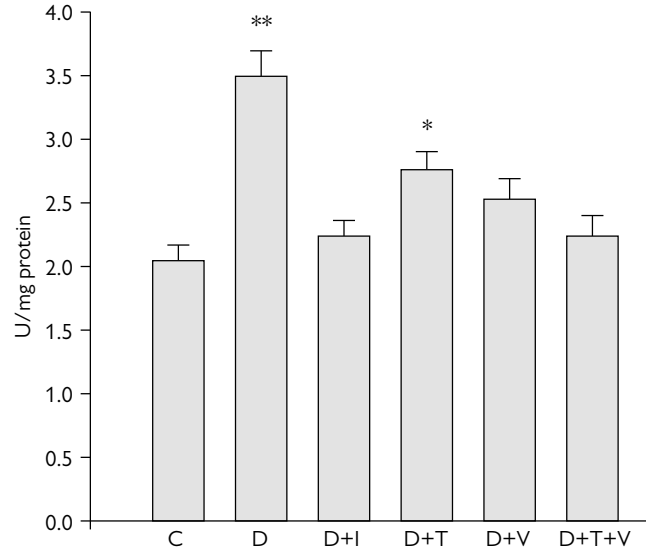

*Figure 1 – Changes in the activity of MAO in brain of control (C), diabetic (D) and diabetic treated rats with insulin (D+I), TSP (D+T), SOV (D+V) and combined dose of TSP and SOV (D+T+V) after three weeks of treatment. Each value is a mean of ± SEM of five or more separate experiments. Fisher's p-values are: **p<0.01, *p<0.05. One unit of enzyme activity is one µmole 4-hydroxyquinoline produced per mg protein per minute at 37 °C.*

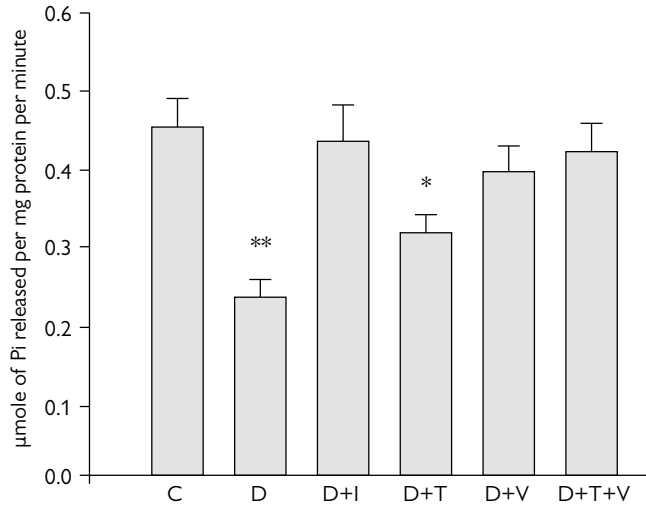

*Figure 2 – Changes in the activity of Ca^{2+}ATPase in brain of control (C), diabetic (D) and diabetic treated rats with insulin (D+I), TSP (D+T), SOV (D+V) and combined dose of TSP and SOV (D+T+V) after three weeks of treatment. Each value is a mean of ± SEM of five or more separate experiments. Fisher's p-values are: **p<0.01, *p<0.05. One unit of enzyme activity is as one µmole of Pi released per mg protein per minute.*

Ca^{2+}ATPase in diabetic rats. There was a significant changes in D+T group ($p<0.05$) with respect to control group. The combined treatment of Ca^{2+}ATPase activity with TSP and SOV for three weeks was found to be the most effective treatment for restoration. Results are summarized in Figure 2.

AChE activity

The activity of AChE of diabetic animals and treated with antidiabetic compounds in synaptosomes after three weeks are shown in Figure 3. The enzyme showed markedly decreased activity ($p<0.001$) in the diabetic brain in comparison with the activity observed in control rats. The activities of AChE of diabetic animals were decrease in D+T ($p<0.01$), D+V ($p<0.05$), and D+T+V group of rats when compared to untreated diabetic rats. The combined treatment with TSP and SOV (D+T+V) for three weeks was found to be the most effective treatment for restoration.

Effects of antidiabetic compounds on lipid peroxidation

The changes in formation of 4-HNE in brain synaptosomes of control, diabetic and diabetic rats after three weeks of treatment with antidiabetic compounds, were measured and results shown in Figure 4. After three weeks of insulin withdrawal, the diabetic rats showed a significant increase ($p<0.01$) in the 4-HNE formation in the rat brain. The formation of 4-HNE of diabetic animals were decrease in D+T,

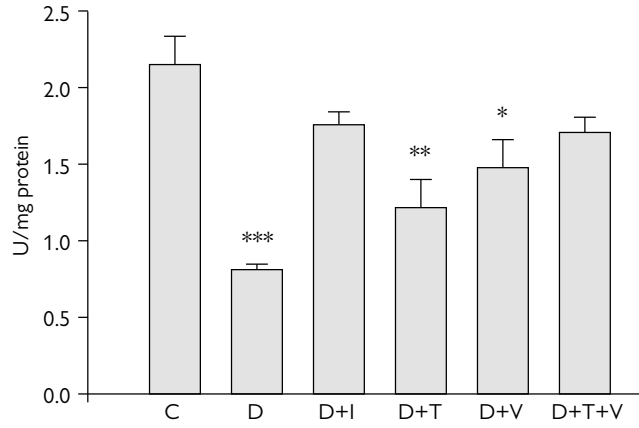

*Figure 3 – Changes in the activity of AChE in brain of control (C), diabetic (D) and diabetic treated rats with insulin (D+I), TSP (D+T), SOV (D+V) and combined dose of TSP and SOV (D+T+V) after three weeks of treatment. Each value is a mean of ± SEM of five or more separate experiments. Fisher's p-values are: ***p<0.001, **p<0.01, *p<0.05. The specific activity of the enzyme is expressed as μmoles of thiocholine produced per min per gram of protein (μmoles/min/protein) at room temperature.*

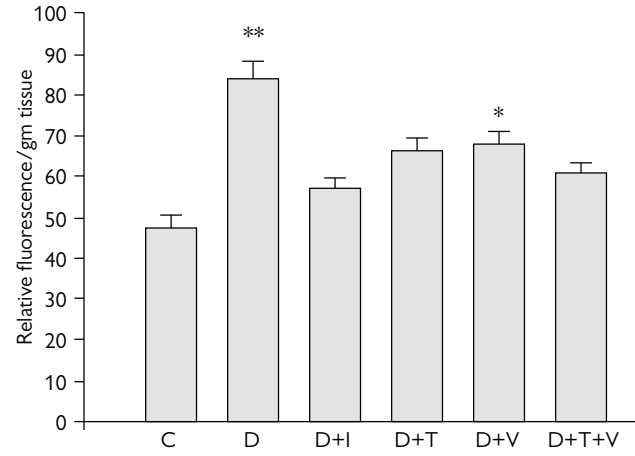

*Figure 4 – Lipid peroxides, 4-HNE content in brain control (C), diabetic (D) and diabetic treated rats with insulin (D+I), TSP (D+T), SOV (D+V) and combined dose of TSP and SOV (D+T+V) after three weeks of treatment. Each value is a mean of ± SEM of five or more separate values from two to three experiments. The comparisons of experimental values are with the controls. Fisher's p-values are: **p<0.01, *p<0.05. Amount of 4-HNE was expressed as percentage fluorescence of control from 1 ml of 10% homogenate (w/v). One unit of lipid peroxidation is defined as percentage changes are calculated taking control as 100%.*

D+V, and D+T+V group of rats when compared to untreated diabetic rats. There was a significant changes in D+V group ($p<0.05$) with respect to control group. The combined treatment of 4-HNE with TSP and SOV (D+T+V) for three weeks was found to be the most effective treatment for restoration.

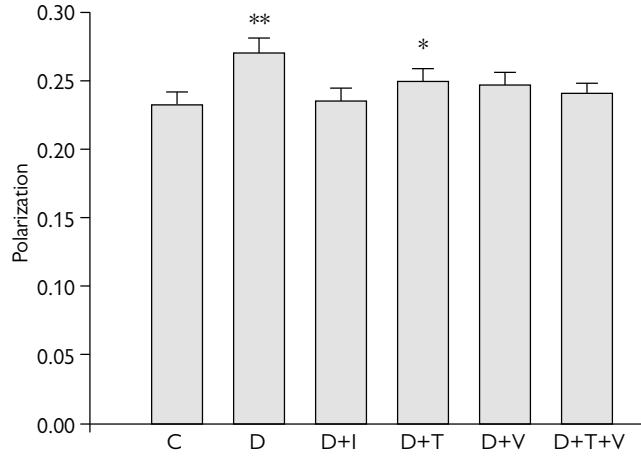

*Figure 5 – Changes in membrane fluidity in brain control (C), diabetic (D) and diabetic treated rats with insulin (D+I), TSP (D+T), SOV (D+V) and combined dose of TSP and SOV (D+T+V) after three weeks of treatment. Each value is a mean of ± SEM of five or more separate values from two to three experiments. Fisher's p-values are: **p<0.01, *p<0.05.*

Effects of antidiabetic compounds on membrane fluidity

The fluidity parameters were determined at 37 °C using 1,6-diphenyl-1,3,5-hexatriene as the probe. The polarization (P) values were found to be higher in three weeks diabetic group than in control group, indicating significant ($p<0.01$)

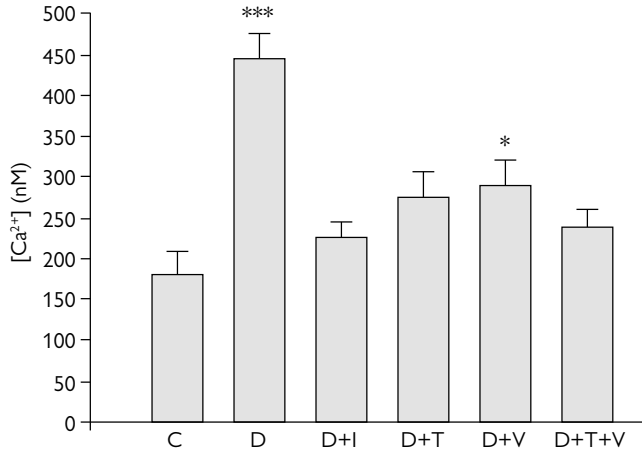

*Figure 6 – Changes in intrasynaptosomal calcium levels in brain control (C), diabetic (D) and diabetic treated rats with insulin (D+I), TSP (D+T), SOV (D+V) and combined dose of TSP and SOV (D+T+V) after three weeks of treatment. Each value is a mean of ± SEM of five or more separate values from two to three experiments. The comparisons of experimental values are with the controls. Statistical significance: ***p<0.001, *p<0.05.*

decreases in fluidity of the membrane in the diabetic group. Polarization (P) values of the diabetic animals were decrease in D+T, D+V, and D+T+V group of rats when compared to untreated diabetic rats. There was a significant reversed the increased fluidity in D+T group ($p<0.05$) with respect to control group. The combined treatment of membrane fluidity with 5% TSP and low dose of SOV in (D+T+V) group for three weeks was found to be the most effective in controlling the diabetic induced changes in membrane fluidity. Results are summarized in Figure 5.

Effects of antidiabetic compounds on calcium levels

The Ca^{2+} levels showed markedly higher ($p<0.001$) in the diabetic brain synaptosomes in comparison with control rats. The Ca^{2+} levels of diabetic animals were decrease in D+T, D+V, and D+T+V group of rats when compared to untreated diabetic rats. There was a significant decreased in D+V group ($p<0.05$) with respect to control group. The combined treatment with TSP and SOV (D+T+V) group for three weeks was found to be the most effective treatment for restoration. Results are shown in Figure 6.

Effects of antidiabetic compounds on neurolipofuscin

The neurolipofuscin deposition increased with diabetes in cerebral hemispheres, as compared to the control animals. Treatment of diabetic animals with D+T, D+V, and D+T+V decrease neurolipofuscin deposition in neurons and also showed

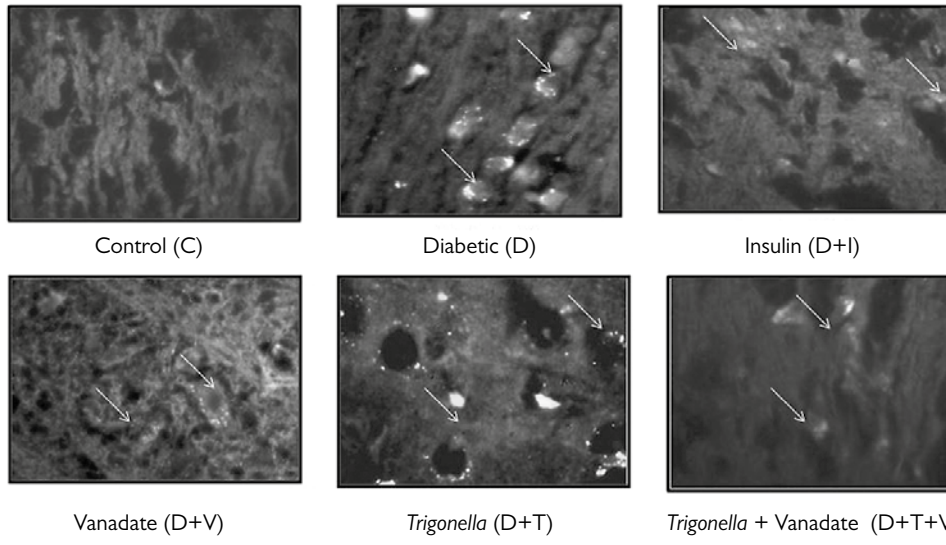

Figure 7 – The neurolipofuscin accumulation (yellow autofluoresence shown by white arrowheads) in cerebral hemispheres of control (C), diabetic (D) and diabetic treated rats with insulin (D+I), TSP (D+T), SOV (D+V) and combined dose of TSP and SOV (D+T+V) after three weeks of treatment.

an increase in the number of neurons without neurolipofuscin when compared with the controls. The combined treatment with low dose of SOV and 5% TSP in (D+T+V) group for three weeks was found to be the most effective treatment for decreasing neurolipofuscin accumulations in diabetic rat brain. The results are presented in the Figure 7.

Discussion

Hyperglycemia has been shown to generate free radicals from auto-oxidation of glucose, formation of advance glycation end products (AGEs) and increased polyol pathway, with concomitant increase in cellular lipid peroxidation and damage of membrane in diabetes.

Trigonella foenum graecum seed powder and vanadate has been earlier shown to have hypoglycemic and antihyperglycemic properties besides other medicinal properties (Mohammad et al., 2006; Baquer et al., 2009, 2011; Kumar et al., 2012a, b, c).

In the present study we examined the activities of membrane linked enzyme (MAO, AChE, Ca^{2+}ATPase), with intracellular calcium levels, lipid peroxidation, membrane fluidity and neurolipofuscin accumulations in brain of alloxan diabetic rats, and to studied whether the co-administration of TSP and SOV prevented and restored the alterations of neuronal markers occurring during diabetes. The three weeks of treatment with insulin, TSP, SOV, and TSP in combination with SOV result in a marked reduction in hyperglycemia in the diabetic rats. The combined treatment (D+T+V) with SOV and TSP was found to be the most effective treatment for diabetes.

Oxidative stress and modulation of antioxidant enzymes may contribute to the deleterious consequence of diabetes mellitus and to the effect of chronic stress in the brain (Baquer et al., 2009). Increased oxidative stress, which contributes substantially to the pathogenesis of diabetic complications, is the consequences of either enhanced reactive oxygen species (ROS) production or attenuated ROS scavenging capacity. The antioxidant defence system and antioxidant enzymes showed lower activities in brain during diabetes rats, this agrees well with the earlier published data (El-Missiry et al., 2004; Baquer et al., 2011; Kumar et al., 2011, 2012b, c). In the present results it was observed that the production of 4-HNE, a product of lipid peroxidation, was significantly increased in brain of diabetic rats as reported earlier (El-Missiry et al., 2004). These increased lipid peroxide formation disturbs the anatomical integrity of the membrane leading to inhibition of several membrane bound enzymes including Ca^{2+}ATPase and MAO. Insufficient activity of Ca^{2+}ATPase, MAO and AChE has been suggested as a contributing factor in the development of diabetic neuropathy (Baquer et al., 2009).

A significant increase in the level of lipid peroxidation in diabetic rats could be due to several mechanism occurring in the diabetic condition like increased generation of free radicals by several mechanisms, including direct glucose auto-

oxidation, non-enzymatic protein glycation, activation of NAD(P)H oxidases and xanthine oxidase (Desco et al., 2002). Diabetic rats exhibited an increased level of lipid peroxidation. Treatment of diabetic rats with insulin, TSP, SOV and a combined therapy of lower dose of SOV with TSP revived normoglycemia and restored the altered level lipid peroxidation.

It is well known that some neurodegenerative processes might preferentially affect the brain as a result of production of free radicals associated with catecholamines metabolism. Mayanil et al. (1982) reported that prolonged diabetes might increase the formation of several biogenic aldehydes and subsequently MAO activity. The present results show the marked increase in MAO activity during diabetes. It has been documented that H_2O_2 derived from MAO activity, represents another source of oxidative stress in brain (Soto-Otero et al., 2001). The treatment of diabetic animals with insulin, SOV and TSP revert the increased activity of MAO. However, TSP treatment partially restored the elevated MAO activity. Combined treatment of 0.2 mg/ml SOV and 5% TSP (D+T+V) completely normalized the increased activity of MAO.

Acetylcholinesterase is one of the important membrane bound enzyme in brain that influence the acetylcholine levels. Several earlier studies reported that, a decrease in AChE level in diabetic male rats with significant increase in lipid peroxidation in brain tissue (Rao et al., 2007). Ghareeb and Hussen (2008) had shown that vanadium improves AChE activity in alloxan diabetic rats. In present study, combined treatment with SOV and TSP to diabetic rats leads to reversal changes of AChE activity close to control rats. TSP and SOV, decreased lipid peroxidation and membrane damage in diabetic rat brain, which leads to restore AChE activity.

In the present study, Ca^{2+}ATPase activity was decreased in brain of diabetic rats. The present findings are in agreement with previous observations showing that the membrane abnormalities in Ca^{2+}ATPase activity led to the occurrence of intracellular calcium overload in experimental rat models of diabetes (Doğru et al., 2005). The decrease in Ca^{2+}ATPase enzyme activity in brain tissue of diabetic animals could also be due to the excessive non-enzymatic glycation of the enzyme itself and/or calmodulin (Baquer et al., 2009). The reduction in the activity of Ca^{2+}ATPase observed in diabetic tissue may be due to the membrane peroxidative damage induced by increased lipid peroxidative status and/or altered antioxidant enzymes of diabetic rats (Lehotsky et al., 2002). In present study, treatment of the experimental animals with SOV (0.6 mg/ml) and TSP seed powder (5% w/w) significantly normalized the activity of Ca^{2+}ATPase near to control values in diabetic rats. Low doses (0.2 mg/ml) of SOV with 5% TSP were the most effective treatment to control the changed in Ca^{2+}ATPase eliciting minimum toxic effects. Kumar et al. (2012a) had shown that treatment alone with 5% dose of TSP significantly changes the increased levels of calcium to normalize levels in diabetic rats.

In the present study, membrane fluidity decreased in diabetes animals. This result agrees with earlier published report (Hong et al., 2004; Kumar et al., 2012a, b). The decreased in membrane fluidity of diabetic brain could be due the peroxidation of membrane phospholipids through free radicals, which is generated by persistent hyperglycemia (Baquer et al., 2011).

Oxidative damage to various brain regions contribute to morphological abnormalities in diabetes. The formation of neurolipofuscin content was increased in experimental diabetes as reported earlier (Sugaya et al., 2004; Kumar et al., 2012a), suggesting that diabetes could have functional change in the neural tissue. Inhibition of neurolipofuscin content and restoration of membrane fluidity by SOV and TSP strongly suggests anti-lipidperoxidative and antidiabetic properties in animals (Kumar et al., 2012a, c). Combined treatment with low dose of SOV and TSP was most effective for restoration of altered membrane fluidity and neurolipofuscin accumulation in diabetic rat brain.

Treatment of the diabetic animals with antidiabetic compounds like insulin, SOV, TSP and combined therapy of TSP with lower dose of SOV restored the high blood glucose levels to control values, altered activities of AChE, Ca^{2+}ATPase, MAO, decreased lipid peroxidation and normalized membrane fluidity in the brain tissue of alloxan diabetic rats.

The beneficial effects observed might be attributed to their hypoglycemic action or insulin mimetic effect of sodium orthovanadate. The insulinotropic property of 4-hydroxisoleucine, an amino acid extracted from *Trigonella foenum graecum* suggests the involvement of insulin secretion modulation in its therapeutic action (Basch et al., 2003; Fuller and Stephens, 2015). SOV treatment was more effective in normalizing these various parameters as compared to TSP given alone, but resulted in a significant weight loss of the treated animals. Therefore, an attempt was made to prevent the toxic effects of vanadium by reducing the dose of SOV administration to 0.2 mg/ml and combining it with TSP treatment to the diabetic animals.

Conclusion

The data shown in this communication conclude that SOV and TSP administration to diabetic rats effectively normalized some of the diabetic aberrations in the brain. Therefore, the dose of vanadate was reduced without compromising with its antidiabetic potential by combining it with TSP. This combined treatment of lower dose of SOV and TSP was found to be most effective treatment in stabilizing the antioxidant defense system and normalizing the membrane functions; therefore the combined therapy can be considered a better alternative to be explored further as a means of diabetic control.

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